APPENDIX
U.S. Serial No. 09/154,903
Pending Claims

8. A preparation of dendritic cells having at least two cell surface markers selected from the group consisting of CD1a, HLA-DR and CD86, produced by contacting hematopoietic stem or progenitor cells with flt3-ligand.

9. (twice amended) A dendritic cell preparation according to claim 8 produced further by contacting the hematopoietic stem or progenitor cells with one or more compounds selected from the group consisting of GM-CSF, IL-4, TNF-α, IL-3, c-kit ligand, fusions of GM-CSF and IL-3, CD40 antibody and CD40 ligand.

10. (twice amended) A dendritic cell population produced by the process of :

(a) contacting hematopoietic stem or progenitor cells with flt3-ligand in an amount sufficient to generate a dendritic cell population;

(b) transfecting the dendritic cells with a gene encoding an antigen; and

(c) allowing the dendritic cells to process and express the antigen.

11. (twice amended) A dendritic cell population according to claim 10 wherein the process further comprises contacting the hematopoietic stem or progenitor cells with one or more compounds selected from the group consisting of GM-CSF, IL-4, TNF-α, IL-3, c-kit ligand, fusions of GM-CSF and IL-3, CD40 antibody and CD40 ligand.

12. (withdrawn) A method of preparing a dendritic cell population comprising the steps of:

(a) contacting hematopoietic stem or progenitor cells with flt3-ligand in an amount sufficient to generate a dendritic cell population;

(b) either (i) exposing the dendritic cells to an antigen-specific peptide or (ii) transfecting the dendritic cells with a gene encoding an antigen-specific peptide; and

(c) allowing the dendritic cells to process and express the antigen.

13. (withdrawn) A method according to claim 12, wherein step (a) further comprises contacting the hematopoietic stem or progenitor cells with a molecule selected from the group consisting of GM-CSF, IL-4, TNF-α, IL-3, c-kit ligand, fusions of GM-CSF and IL-3, and CD40 binding protein.

15. (new) A dendritic cell population produced by the process of :

(a) contacting hematopoietic stem or progenitor cells with flt3-ligand in an amount sufficient to generate a dendritic cell population;

(b) exposing the dendritic cells to an antigen; and

(c) allowing the dendritic cells to process and express the antigen.

16. (new) A dendritic cell population according to claim 15 wherein the process further comprises contacting the hematopoietic stem or progenitor cells with one or more compounds selected from the group consisting of GM-CSF, IL-4, TNF-α, IL-3, c-kit ligand, fusions of GM-CSF and IL-3, CD40 antibody and CD40 ligand.

17. (new) The dendritic cell preparation according to claim 8 containing recombinant human flt3-ligand.

18. (new) The dendritic cell preparation according to claim 9 containing recombinant human flt3-ligand.

19. (new) The dendritic cell preparation according to claim 10 wherein the flt3-ligand is a recombinant human flt3-ligand.

20. (new) The dendritic cell preparation according to claim 11 wherein the flt3-ligand is a recombinant human flt3-ligand.

21. (new) The dendritic cell preparation according to claim 15 wherein the flt3-ligand is a recombinant human flt3-ligand.

22. (new) The dendritic cell preparation according to claim 16 wherein the flt3-ligand is a recombinant human flt3-ligand.

23. (new) The dendritic cell preparation according to claim 18 containing recombinant human GM-CSF.

24. (new) The dendritic cell preparation according to claim 20 wherein the compound is a recombinant human GM-CSF.

25. (new) The dendritic cell preparation according to claim 22 wherein the compound is a recombinant human GM-CSF.

26. (new) The dendritic cell preparation according to claim 18 containing CD-40 ligand.

27. (new) The dendritic cell preparation according to claim 20 wherein the compound is CD-40 ligand.

28. (new) The dendritic cell preparation according to claim 22 wherein the compound is CD-40 ligand.

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Immunology

Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*

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ABSTRACT **Dendritic cells (DCs) are unique in their ability to stimulate T cells and initiate adaptive immunity. Injection of mice with the cytokine Flt3-ligand (FL) dramatically expands mature lymphoid and myeloid-related DC subsets. In contrast, injection of a polyethylene glycol-modified form of granulocyte/macrophage colony-stimulating factor (GM-CSF) into mice only expands the myeloid-related DC subset. These DC subsets differ in the cytokine profiles they induce in T cells *in vivo*. The lymphoid-related subset induces high levels of the Th1 cytokines interferon γ and interleukin (IL)-2 but little or no Th2 cytokines. In contrast, the myeloid-related subset induces large amounts of the Th2 cytokines IL-4 and IL-10, in addition to interferon γ and IL-2. FL- or GM-CSF-treated mice injected with soluble ovalbumin display dramatic increases in antigen-specific antibody titers, but the isotype profiles seem critically dependent on the cytokine used. Although FL treatment induces up to a 10,000-fold increase in ovalbumin-specific IgG2a and a more modest increase in IgG1 titers, GM-CSF treatment favors a predominantly IgG1 response with little increase in IgG2a levels. These data suggest that distinct DC subsets have strikingly different influences on the type of immune response generated *in vivo* and may thus be targets for pharmacological intervention.**

Immune responses against T-cell-dependent antigens are heterogeneous with respect to the cytokines made by T-helper cells and the class of antibody secreted by B cells (1–5). Immune responses dominated by $CD4^+$ T cells producing interferon (IFN) γ and B cells secreting IgG2a antibody are termed "Th1" responses; those dominated by $CD4^+$ T cells producing interleukin (IL)-4 and IL-10 and B cells secreting IgG1 are termed "Th2" responses. At the individual T-cell level, considerable heterogeneity of cytokine profiles can be seen with T-cell clones, raising the possibility that the Th1 and Th2 global phenotypes may only represent two polar extremes of all possible single cell phenotypes (4). The type of cytokine produced early in an immune response appears to be key in determining whether a Th1-like or Th2-like immune response is generated. Thus, early production of IL-4 induces further IL-4 production in T cells whereas IL-12 induces IFNγ in T cells (1–5). However, the question of which cell types initiate the polarization of the response is less clear. The antigen-presenting cell, of which dendritic cells (DCs) are prime examples (6, 7), is thought to play a key role in determining the type of immune response (1–5). DCs, through production of IL-12 (8–10) may preferentially direct the development of Th1 cells *in vitro* (8) and *in vivo* (11) whereas other cells, such as B cells (12, 13) and NK T cells (14), may influence the development of Th2 responses.

Attempts to study the regulation of immune responses by DCs have been impeded by their rarity in tissues. A recent solution to this problem has been the identification of DC growth factors such as Flt3-ligand (FL), which induce a profound expansion of mature DC subsets in mice (15, 16). The mature DC subsets generated in FL-treated mice all express high levels of CD11c and major histocompatibility complex class II, as well as CD86 and CD40, but differ in their levels of CD11b expression (15, 16) (Fig. 1). The majority of cells within the $CD11c^+$ $CD11b^{dull/-}$ subset express high levels of CD8α and DEC-205, markers that are expressed on lymphoid-related DC subset in mice (17–21). Lymphoid-related DC have been shown to down-regulate the activation of T cells *in vitro* (20, 21) through a Fas-dependent mechanism (20). Cells within the $CD11c^+$ $CD11b^{bright}$ subset do not express CD8α or DEC-205 but do express F4–80, 33D-1, and other myeloid-related markers, suggesting that they might be of myeloid origin (15, 16). These subsets are localized in distinct microenvironments, with the lymphoid-related subset residing in the T-cell zones and the myeloid-related subset residing in the marginal zones of the spleen (16, 21). Both subsets appear equally competent at stimulating antigen-specific T cell proliferation *in vitro* (E.M., B.P., C.M., K.B., E. Daro, M. Teepe, and H. McKenna, unpublished work). However, the lymphoid-related population can be induced to secrete much higher levels of biologically active IL-12 than the myeloid-related population (16), consistent with recent observations in normal mice (22). In this study, we investigate the ability of these DC subsets to prime antigen-specific T cells *in vivo* and examine the cytokine profiles that they induce in T cells.

MATERIALS AND METHODS

Mice. All mice were housed in a specific-pathogen-free facility. BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory; a male DO11.10/SCID mouse was received from the laboratory of Marc Jenkins (University of Minnesota) and was bred to BALB/c/SCID mice in house. For adoptive transfers, age- and sex-matched recipients were injected i.v. with 2×10^6 T cells as described (23).

Injections. Mice were injected s.c. with 10 μg of recombinant human FL (human Chinese hamster ovary cell-derived produced at Immunex) for 9 consecutive days. Recombinant granulocyte/macrophage colony-stimulating factor (GM-CSF) was modified with polyethylene glycol at Immunex and was injected s.c. (5 μg per day) for 5 consecutive days. In adjuvant studies, chicken ovalbumin (Sigma) was freshly prepared in PBS, was filtered, and was injected s.c.

DCs. DC subsets from FL- and GM-CSF-treated mice were sorted by flow cytometry as described (15, 16). DCs were incubated with ovalbumin 323–339 (OVA[323–339]) peptide at 4°C for 2 hours, were washed, and were injected into the footpads of reconstituted mice (3×10^5 DCs per footpad).

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Abbreviations: IFN, interferon; IL, interleukin; DC, dendritic cell; FL, Flt3-ligand; TCR, T cell receptor; GM-CSF, granulocyte/macrophage colony-stimulating factor.

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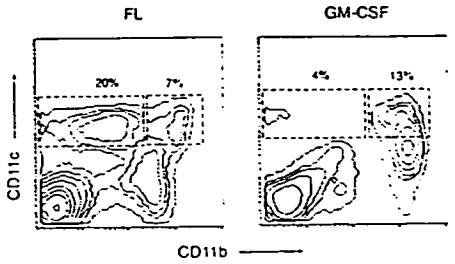

FIG. 1. Relative expansion of DC subsets from FL- and GM-CSF-treated mice. Shown is the distribution of CD11c and CD11b on spleen cells from cytokine treated mice. $CD11c^+$ $CD11b^{bright}$ (myeloid-related) and $CD11c^+$ $CD11b^{dull/-}$ (lymphoid-related) subsets of DCs are defined as shown. Note that, in FL-treated mice, the lymphoid-related DCs are more prevalent than the myeloid-related DCs. Similar profiles are obtained with both C57BL/6 and BALB/c strains. Data are representative of a large number of experiments.

In Vitro Cultures. Popliteal lymph node cells (5×10^5 cells) were plated in triplicate in 200 μl of DMEM complete supplemented with 5% fetal bovine serum, together with different concentrations of OVA[323–339]. Proliferative responses were assessed after 72 hours of culture in a humidified atmosphere of 5% CO_2 in air. Cultures were pulsed with 0.5 μCi tritiated thymidine [^{3}H] for 5 hours, and the cells were harvested onto glass fiber sheets for counting on a gas-phase β counter. For cytokine assays, aliquots of culture supernatants were removed after 72 hours and were assayed for the presence of IFNγ, IL-2, IL-4, and IL-10 by ELISAs adapted from PharMingen protocols.

Measurement of Ovalbumin-Specific Serum Titers. Ninety-six-well ELISA plates (Maxisorp, Nunc) were coated overnight with 1 μg/well ovalbumin in PBS at 4°C, were blocked with PBS/5% fetal bovine serum, and washed with PBS/0.1% Tween-20. Serum samples were diluted in PBS/5% fetal bovine serum (starting at 1/100), and threefold dilutions were made. Plates were incubated for 2 hours at room temperature, were washed, and were incubated with alkaline phosphatase-conjugated anti-IgG1 (1/2,000; PharMingen), anti-IgG2a, anti-IgG2b, or anti-IgM (1/1,000; PharMingen) detecting antibodies for an additional 2 hours at room temperature. Plates were washed again, and enzyme activity was detected with p-nitrophenyl phosphate disodium (Sigma). The amount of reaction product was assessed on an ELISA plate reader at an OD of 405 nm by using the DELTASOFT program (DeltaPoint, Monterey, CA). Multiple-point analysis was performed on each set of isotype titrations by using the BIOASSAY program (Immunex), selecting a maximum value for each isotype and determining for each sample the dilution giving half-maximal OD value, thus generating arbitrary unit-per-milliliter values as described (24).

RESULTS

Lymphoid and Myeloid-Related DCs from FL-Treated Mice Prime T Cells *in Vivo* Equally Efficiently. To investigate the ability of these DC subsets to prime antigen-specific T cells *in vivo*, we used T-cell receptor (TCR) transgenic mice that contained rearranged TCRα and TCRβ genes in their germline DNA encoding a TCR specific for the peptide fragment OVA [323–339] bound to I-Ad class II major histocompatibility complex molecules (DO11.10 mice) (25). The transgenic TCR could be detected with the KJ126 mAb that binds only to this particular TCR heterodimer (26). Because DO11.10 T cells contain a small subpopulation of cells with a memory phenotype, presumably because of activation through a second endogenously rearranged TCR (27), all of our experiments were performed with DO11.10/SCID mice, which do not contain cells with this memory phenotype (26). TCR transgenic T cells from DO11.10/SCID mice were adoptively transferred into syngeneic BALB/c recipients, such that they constituted a small (<0.3%) but detectable proportion of all T cells as described (23, 24, 28).

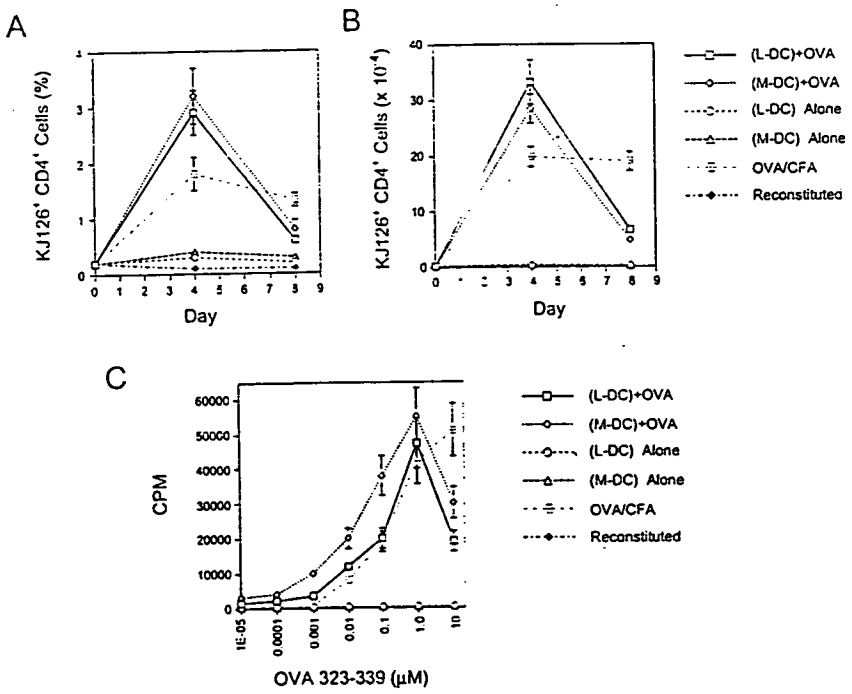

FIG. 2. (*A* and *B*) Kinetics of expansion of antigen-specific ($KJ126^+$ $CD4^+$), transgenic T cells in mice primed with lymphoid-related (L-DC: $CD11c^+$ $CD11b^{dull/-}$) and myeloid-related (M-DC; $CD11c^+$ $CD11b^{bright}$) DC subsets from FL-treated mice. Sorted DCs were loaded with OVA[323–339] *in vitro* and were injected into the footpads of mice reconstituted with transgenic T cells. T-cell expansion was measured by flow cytometric analysis of lymph node cells at various time points. Data were pooled from six independent experiments with a total of 18–21 lymph nodes per time point. (*C*) *In vitro* restimulation of antigen-specific T cells expanded *in vivo*, with varying concentrations of OVA[323–339] peptide. *In vitro* lymph node cultures were set up 4 days after *in vivo* priming, were pulsed with [H]^3 72 hrs later, and were mashed after 5 hrs.

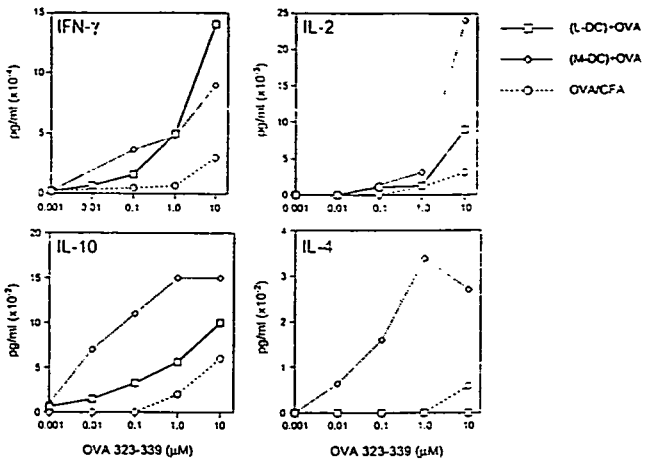

FIG. 3. Cytokine production by antigen-specific T cells restimulated *in vitro* with varying concentrations of OVA[323–339]. *In vitro* lymph node cultures were set up 4 days after priming, and supernatants were assayed 72 hours later by cytokine ELISA. Data are representative of seven independent experiments.

Lymphoid-related DC and myeloid-related DC subsets from spleens of FL-treated mice were isolated by flow cytometry, were loaded with OVA[323–339] *in vitro*, and were injected into the footpads of T-cell-reconstituted mice. The ensuing $CD4^+$ OVA-specific T-cell response was monitored by flow cytometry. Both DC populations could prime T cells in an antigen-specific manner (Fig. 2 *A* and *B*), with similar kinetics and magnitude. Thus, 4 days after priming, at the height of the immune response, the percentage of $KJ126^+$ $CD4^+$ T cells was ≈3% in the draining lymph nodes of mice primed with either the lymphoid or myeloid DC subset; by day 8, the percentage in both groups of mice had almost returned to baseline levels. The absolute numbers of $KJ126^+$ $CD4^+$ T cells in the draining lymph nodes reflect this kinetics.

At the height of the response, 4 days after priming, the popliteal lymph nodes were harvested, and single-cell suspensions were prepared and cultured *in vitro* with various concentrations of OVA[323–339] to assess the *in vitro* proliferative capacity of antigen-specific T cells. T cells that had been primed *in vivo* by either the lymphoid- or myeloid-related DCs could proliferate efficiently *in vitro* on restimulation with antigen; however, there was a 2- to 5-fold increase in the sensitivity of antigen-specific T-cell proliferation in T cells that had been primed *in vivo* with the myeloid DC, compared with those primed with the lymphoid-related DC subset (Fig. 2*C*).

Lymphoid- and Myeloid-Related DCs from FL-Treated Mice Exert Differential Effects on Cytokine Production in Antigen-Specific T Cells *in Vivo*. Cytokine production by antigen-specific T cells primed by the lymphoid or myeloid-related DC subsets was measured by assaying the culture supernatants from the cultures described above for IFNγ, IL-2, IL-10, and IL-4. Assessment of cytokine production in these cultures revealed that there were no significant differences in IFNγ and IL-2 production in T cells primed by either DC subset; however, the myeloid-related DC subset always induced much greater levels of IL-10 and IL-4 production (Fig. 3). Thus, the lymphoid-related subset induced no detectable IL-4 whereas the myeloid-related subset induced high levels of IL-4. Furthermore, the T cells primed by the myeloid-related DC subset exhibited a >100-fold increase in the antigen dose-dependent sensitivity of IL-10 production, compared with T cells primed by the lymphoid-related DC subset.

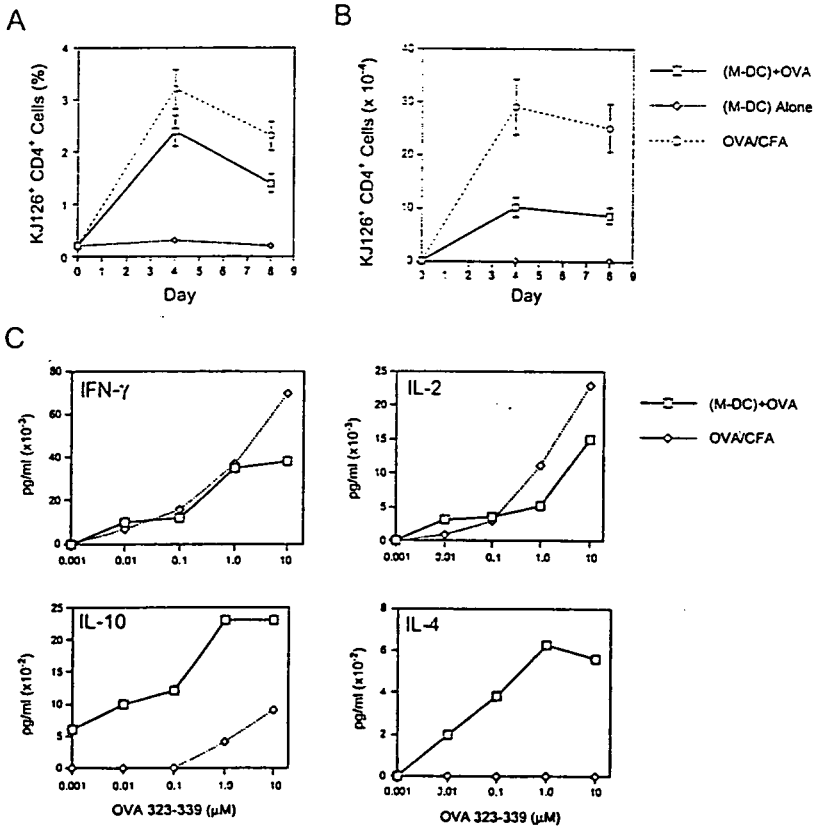

FIG. 4. (*A* and *B*) Kinetics of expansion of antigen-specific ($KJ126^+$ $CD4^+$), transgenic T cells in mice primed with the myeloid DC (M-DC) subset in GM-CSF-treated mice. (*C*) Cytokine production by antigen-specific T cells restimulated *in vitro* with varying concentrations of OVA[323–339]. *In vitro* cultures were set up 4 days after priming, and supernatants were assayed 72 hours later. Data are representative of two independent experiments.

GM-CSF Treatment Preferentially Expands a Myeloid-Related DC Subset, Which Resembles the Myeloid DC in FL-Treated Mice. Administration (5 days) of a chemically modified (pegylated) form of GM-CSF (which exhibits an extended biological half-life, compared with underivatized GM-CSF) into mice preferentially expands the myeloid-related DC ($CD11c^+$ $CD11b^{int.}$) subset (Fig. 1). This myeloid-related DC subset appears to be similar to that generated by FL-treatment, in so far as it failed to express detectable CD8α or DEC-205 (E.M., B.P., C.M., K.B., E. Daro, M. Teepe, and H. McKenna, unpublished work), which have been shown to be expressed selectively on lymphoid-related DC (15, 16, 19). They are also able to prime naive, antigen-specific T cells *in vitro* as efficiently as the myeloid or lymphoid-related DC subsets from FL-treated mice (E.M., B.P., C.M., K.B., E. Daro, M. Teepe, and H. McKenna, unpublished work).

Similiar experiments with the sorted DC subset from GM-CSF-treated mice revealed that this putative myeloid-related population also could prime OVA-specific T cell expansion as efficiently as the lymphoid and myeloid DC populations from FL-treated mice (Fig. 4 *A* and *B*). As observed previously with the myeloid-related population from FL treated mice, there was significant production of IL-4 and IL-10 by the OVA-specific T cells after restimulation with OVA[323–339] *in vitro* (Fig. 4*C*). These data support the notion the distinct DC subsets prime T cells equally efficiently but elicit different cytokine profiles in antigen-specific T cells *in vivo*.

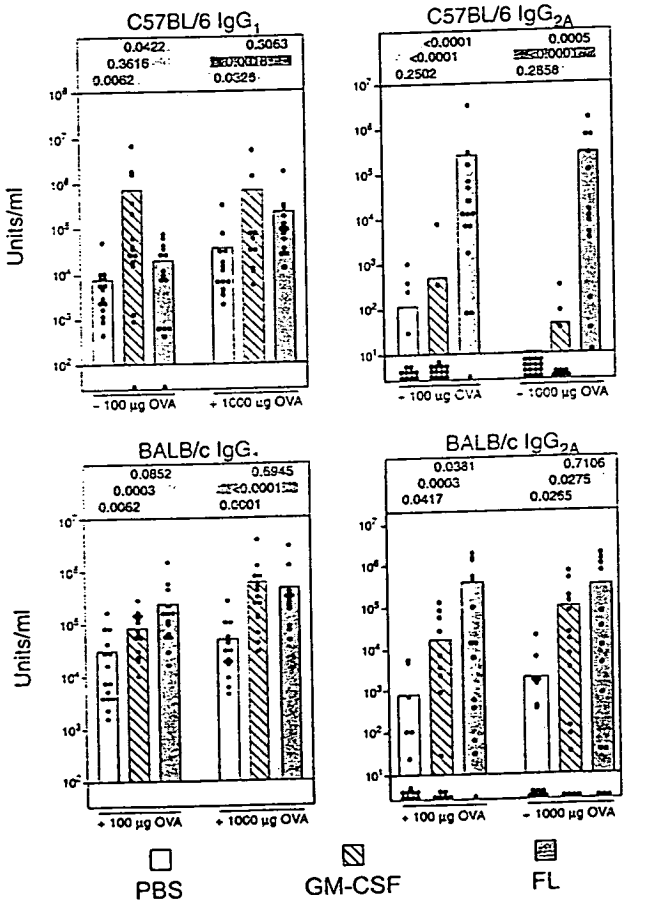

FIG. 5. Adjuvant effects of FL and GM-CSF in ovalbumin-immunized mice. Shown are antigen-specific, secondary serum antibody titers in FL- and GM-CSF-treated mice after challenge with ovalbumin. PBS- or cytokine-treated mice were primed and boosted with ovalbumin, and secondary anti-ovalbumin antibody responses were measured by ELISA. Each dot represents a single mouse. Data are pooled from three independent experiments, each of which had five mice per group. Because the data are not normally distributed, the Wilcoxon rank sum 2-sided test has been used to analyze the *P* values. *P* values comparing the differences between the PBS, GM-CSF, and FL groups are shown above each graph.

FL and GM-CSF Dramatically Enhance Antibody Production to Soluble Antigens but Differ in the Class of Antibodies They Induce. To determine whether these differences in T-cell cytokine profiles could influence the class of antibody produced *in vivo*, we performed the following experiment. Cohorts of BALB/c or C57BL/6 mice were treated with either FL or GM-CSF for 9 and 5 days, respectively, to induce maximal expansion of DCs. The effects of FL and GM-CSF on expanding DC subsets *in vivo* does not appear to depend on the strain of mice used (data not shown). On the final day of cytokine treatment, mice were injected s.c. with various doses of ovalbumin in PBS. Twenty-one days later, identical immunizations were performed with no cytokine treatment. One week later (day 28), mice were bled, and the ovalbumin-specific antibody titers were determined by ELISA. Both FL and GM-CSF significantly enhanced the ovalbumin-specific antibody titers of all isotypes analyzed (Fig. 5) compared with the ovalbumin-immunized, PBS-treated control mice. Consistent with our recent observations (24), FL treatment resulted in a >10,000-fold increase in IgG2a titers but a much more modest increase in IgG1 titers over PBS-treated controls. However, GM-CSF treatment resulted in a much weaker IgG2a response than FL-treatment, and this was more apparent in the C57BL/6 strain, in which GM-CSF failed to stimulate IgG2a (Fig. 5). In contrast, GM-CSF stimulated significant levels of IgG1, and, in some cases, the levels of IgG1 stimulated were significantly higher than those in FL-treated mice.

The data in Fig. 5 are pooled from three independent experiments, each of which had five mice per group. The variability in the IgG2a OVA-specific antibody titers in mice treated with FL or GM-CSF is caused largely by variability between individual experiments. The source of this experiment to experiment variation is not known. Nevertheless, each experiment showed exactly the same trends between groups: compared with GM-CSF- and PBS-treated mice, FL-treated mice not only displayed much larger titers of IgG2a OVA-specific antibody but also had much higher frequency of responders. This is evident from the means of the graphs as well as with differences in the numbers of nonresponders within each group. The nonresponders are stacked at the bottom of the graph for each group. Thus, although both FL and GM-CSF acted as adjuvants, there were class-specific differences in the antibody profiles generated by each cytokine, with FL giving a predominantly IgG2a response and GM-CSF favoring an IgG1 response. These results reflect the differences in cytokine profiles observed in T cells stimulated by the distinct DC subsets.

DISCUSSION

The results presented in this paper suggest that distinct DC subsets may differentially regulate the Th1/Th2 balance of an immune response *in vivo*. Although both the lymphoid and myeloid-related DC subsets can prime antigen-specific T cells equally efficiently, they induce distinct cytokine profiles in T cells. The lymphoid-related DCs induce the Th1 cytokines IFNγ and IL-2 whereas the myeloid-related DC subset induces high levels of the Th2 cytokines IL-4 and IL-10, in addition to the Th1 cytokines.

Mechanism of T-Cell Cytokine Skewing by Distinct DC Subsets. The precise mechanism by which these DC subsets mediate their effects is, at present, unclear but may well relate to potential differences in the repertoire of costimulatory molecules expressed by the subsets. The difference is unlikely to be accounted for by the B7 molecules, by CD40, or by the levels of class II major histocompatibility complex–peptide complexes on the surfaces because the myeloid- and lymphoid-related DC subsets in FL-treated mice express similar levels of all of these molecules (4). However, potential differences in the expression of costimulatory molecules such as OX-40 ligand

[which may stimulate IL-4 production in T cells (29)] may account for the functional dichotomy of the two subsets. Another factor that could contribute to this dichotomy is the cytokines made by the DCs themselves. Lymphoid-related DCs can be induced to secrete higher levels of biologically active IL-12 than myeloid DCs (16, 22). Therefore, the former may have induced greater IFNγ production in T cells than the latter. However, our experiments suggest that even the relatively low levels of IL-12 produced by the myeloid-related DC subset is sufficient to induce abundant IFNγ production in T cells. Clearly the expression of Th2-inducing cytokines such as IL-4 (1) and IL-6 (30) by the DC subsets warrants further investigation.

The question of whether the DC subsets interact directly with the T cells *in vivo* or whether they transfer antigen to a third cell type is intriguing, particularly given the resurgent interest in the phenomenon of "cross-priming" (31, 32). Thus, it is possible that antigen is transferred from the exogenous DC subset to some endogenous APC population, which then may present it to T cells. If the lymphoid and myeloid DC subsets differed in their abilities to hand over antigen to other antigen-presenting cells, this potentially could lead to differences in T-cell priming or in the cytokines made by the T cells.

Adjuvant Effects of FL and GM-CSF. Whatever the mechanism, the differential cytokine skewing in T cells by distinct DC subsets appears to result in strikingly different antibody profiles being elicited by FL and GM-CSF (Fig. 5). In C57BL/6 mice, GM-CSF (which preferentially expands the myeloid DC subset; Fig. 1) elicits significant increases in IgG1 titers but no IgG2a production (Fig. 5). In contrast, FL (which expands both the lymphoid and myeloid DC subsets, at a numeric ratio of ≈3:1; Fig. 1) elicits modest increases in IgG1 titers but dramatic increases in IgG2a titers (Fig. 5). These differences in antibody profiles are consistent with the cytokine profiles induced in T cells. Lymphoid-related DCs would prime T cells to become Th1 T cells, which enhance IgG2a secretion by B cells. Myeloid-related DCs would prime T cells to make abundant IL-4 and IL-10 (in addition to IFNγ and IL-2), and it is likely that the Th2 cytokines would mitigate the effects of IFNγ, thus favoring an IgG1 response. Similar though less striking trends are observed in BALB/c mice. This strain difference may be attributed to potential differences in production of IL-18 (33) or IL-1α by the DC subsets in the different strains. Both IL-18 and IL-1α can potentiate IL-12-induced Th1 development in BALB/c but not C57BL/6 mouse strains (34).

Perspectives. These data are consistent with recent observations (35) that indicate that lymphoid and myeloid DCs in normal mice also can induce distinct cytokine profiles in T cells. Both studies suggest that the lymphoid and myeloid DCs prime T cells equally efficiently *in vivo*. We currently are investigating whether lymphoid-related DCs could tolerize CD4+ T cells *in vivo* via a Fas-dependent mechanism (20). One possibility is that CD4+ T cells may be tolerized *in vivo* by repeated stimulation with lymphoid- but not myeloid-related DC. Indeed, the 2- to 5-fold less efficient *in vitro* stimulation of T cells, which were primed *in vivo* by the lymphoid DCs (Fig. 2*C*), may represent a dampening of the T-cell response by activation-induced cell death. In this context, it is worth noting that activated Th1 cells, rather than Th2 cells, are susceptible to Fas-mediated apoptosis (36, 37). Therefore, the lymphoid and myeloid DC subsets initially may prime T cells equally efficiently, but, on repeated stimulation by the lymphoid DCs, the resulting Th1 T cells may be more susceptible to Fas-mediated apoptosis.

Finally, the functional differences of the DC subsets described here should be viewed in the context of their geographical separation in lymphoid microenvironments. In the spleen, the lymphoid and myeloid DC subsets are localized in the T-cell zones and the marginal zones, respectively (16). The possibility that these subsets may be exposed to different types of antigens because of their geographical isolation and differences in their cell-surface markers must now be investigated.

In summary, our data suggest that the class of immune response generated *in vivo* can be controlled differentially by distinct DC subsets. This may provide a unique opportunity to elicit optimally effective immunity in different clinical settings by using distinct DC subpopulations. Thus, in various autoimmune diseases or in *Helicobacter pylori* infections, the prospect of using myeloid-related DC or GM-CSF to vaccinate against pathogenic Th1 responses may be attractive. In contrast, lymphoid-related DCs that promote protective Th1 responses may hold great promise in tumor immunotherapy, antiviral immunity, and in allergy.

We thank Dr. Marc Jenkins for advice and DO11.10/SCID mice; Dr. Muriel Moser and Roberto Maldanado for sharing unpublished data; Dr. Nancy Nightlinger for synthesis of pegylated GM-CSF; Dr. Alan Alpert, Daniel Hirschstein, and Steve Braddy for help with flow cytometry; Anne Aumell for editorial assistance; Dr. Jacques Banchereau for advice and discussion; and Drs. Ken Shortman, Gus Nossal, Muriel Moser, Doug Williams, Hilary McKenna, and Laurent Galibert for critical comments on the manuscript.

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Generation of murine dendritic cells from flt3 ligand-supplemented bone marrow cultures

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Running title: Dendritic cells from FL-supplemented bone marrow cultures

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Abstract

Murine dendritic cells (DC) can be classified into at least two subsets, "myeloid-related" ($CD11b^{bright}$, $CD8a^-$) and "lymphoid-related" ($CD11b^{dull}$, $CD8a^+$), but the absolute relationship between the two remains unclear. Methods to generate DC from bone marrow (BM) precursors in vitro typically employ GM-CSF as the principal growth factor, and the resultant DC exhibit a myeloid-like phenotype. Here we describe a Flt3-ligand (FL)-dependent BM culture system that generated DC with more diverse phenotypic characteristics. Murine BM cells cultured at high density in recombinant human FL for 9 days developed into small, lymphoid-sized cells, most of which expressed CD11c, CD86, and MHC Class II. The $CD11c^+$ population could be divided into two populations based on the level of expression of CD11b, which may represent the putative myeloid- and lymphoid-related subsets. The FL in vitro-derived DC, when treated with interferon-a (IFNa) or lipopolysaccharide (LPS) during the final 24 hours of culture, expressed an activated phenotype that included upregulation of MHC Class II, CD1d, CD8a, CD80, CD86, and CD40. The FL-derived DC also exhibited potent antigen processing and presenting capacity. Neutralizing anti-interleukin-6 (IL-6) antibody, but not anti-GM-CSF, significantly reduced the number of DC generated in vitro with FL, suggesting that IL-6 has a role in the development of DC from BM precursors. Stem cell factor (SCF), which exhibits some of the same bioactivities as FL, was unable to replace FL to promote DC development in vitro. This culture system will facilitate detailed analysis of murine DC development.
Key words: Flt3 ligand; dendritic cells; interleukin-6; IFNa; antigen presentation.

Introduction

DC capture and process antigens for presentation to naive T cells and B cells.[1] Although they are relatively rare, DC are found in a broad range of lymphoid and non-lymphoid tissues and are considered to be essential for the rapid, efficient initiation of an immune response. DC are considered to arise from either myeloid-committed or lymphoid-committed progenitors[2]; however, the specific stages of development within these lineages are poorly defined, largely due to a lack of understanding of which growth factors regulate this process. More recent studies have demonstrated that at least two cytokines, Flt3 ligand (FL) and GM-CSF, induce DC expansion in vivo.[3-6] Administration of FL to mice results in large increases in vivo of two populations of DC, which appear to represent myeloid- and lymphoid-related subsets in normal lymphoid tissues.[3] In contrast, GM-CSF administration favors in vivo expansion of only the myeloid-related subset.[4] Studies in gene knockout (KO) mice support the notion that these cytokines play different roles in normal DC development. FL-deficient mice have reduced numbers of lymphoid tissue DC.[7] In contrast, mice lacking GM-CSF have normal numbers of DC in lymphoid tissues[8], but are functionally deficient at initiating B cell and T cell responses.[9,10]

The role of cytokines in DC development from hematopoietic precursors may be more easily examined using in vitro culture systems. Inaba et al have shown that murine BM cells cultured in GM-CSF for 6-8 days generate large numbers of mature DC.[11] These GM-CSF-derived DC can be further activated and enriched by including IL-4.[12,13] DC derived in GM-CSF plus IL-4 express cell surface antigens typically associated with DC, including DEC205, MHC Class II, CD80 and CD86, and demonstrate potent allostimulatory activity.[13] Based upon their expression of myeloid cell surface antigens such as CD11b, 33D1, and F4/80, as well as the lack of CD8α expression, these GM-CSF-derived cells are considered to be the progeny of cells of the myeloid lineage. Lymphoid-related DC can be generated by culturing thymus-derived, $CD4^{low}$ precursors in a cytokine combination that includes SCF, IL-1, tumor-necrosis factor-a (TNFa), IL-3, and IL-7, but not GM-CSF.[14] These DC express variable levels of CD11b, and high levels of MHC Class II, as well as CD80 and CD86, but fail to express CD8α, normally found on a subset of DC from lymphoid tissues of mice.[15]

Here we describe a novel method to generate large numbers of DC in vitro from BM cells cultured in FL alone. Upon activation with LPS or IFNa, these FL-derived DC share morphological characteristics and phenotypic cell surface antigens found on normal lymphoid tissue-derived DC subsets. This culture system will allow us to investigate endogenous factors that contribute to DC development in the mouse, and to compare directly the effects of exogenous cytokines on DC development and differentiation.

Materials and Methods

Mice. Female BALB/c, DBA/2 and C57BL/6 mice (8-12 weeks of age) were obtained from Taconic (Germantown, NY). The D011.10 mice were a kind gift from Dr. Mark Jenkins (University of Minnesota). The C57BL/6 IL-6 gene KO and control mice were purchased from The Jackson Laboratory (Bar Harbor, MA). All mice were housed under specific pathogen-free conditions.

Cell preparations. BM cells were isolated by flushing femurs with 2 ml phosphate buffered saline (PBS) supplemented with 2% heat-inactivated fetal bovine serum (FBS) (Gibco BRL Life Technologies, Grand Island, NY). The BM cells were centrifuged once, then resuspended in tris-ammonium chloride at 37°C for 2 minutes to lyse red blood cells. The cells were centrifuged again, then resuspended in culture medium (CM) consisting of McCoy's medium supplemented with essential and non-essential amino acids, 1 mM sodium pyruvate, 2.5 mM HEPES buffer pH 7.4, vitamins, 5.5×10^{-5} M 2-mercaptoethanol (2-ME), 100 units/ml pennicillin, 100 mg/ml streptomycin, 0.3 mg/ml L-glutamine (PSG), and 10% FBS (all media reagents from Gibco).

DC cultures. BM cells were cultured in CM containing 200 ng/ml recombinant human FL (Immunex) for 9 days at 1×10^6/ml, in six-well plates (Costar Corning, Cambridge, MA) unless otherwise noted. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air. DC were harvested from the cultures by vigorously pipetting and removing non-adherent cells, then washing each well 2 times with room temperature phosphate-buffered saline (PBS) without Ca^{++} or Mg^{++} to remove loosely adherent cells, which were pooled with the non-adherent fraction.

DC were also generated in GM-CSF plus IL-4-supplemented BM cultures as described.[16] Briefly, BM was processed as described above including lysis of red blood cells. BM cells were cultured at 4×10^5/ml in CM containing 20 ng/ml murine GM-CSF (Immunex) and 20 ng/ml murine IL-4 (Immunex), in 6-well plates. On days 3 and 5 of culture, plates were swirled gently before removing 2/3 of the conditioned medium with the non-adherent cells. Fresh GM-CSF and IL-4-containing medium was added back to the cultures. These cultures were harvested for the non-adherent fraction after vigorous pipetting of DC clusters on day 7, and consisted of DC and monocytes.

Activation of the DC from FL-supplemented cultures was accomplished by the addition of 10 ng/ml recombinant murine GM-CSF, 1000 units/ml human IFNa A/D (Genzyme, Cambridge, MA), or 1 mg/ml E. coli (0217:B8)-derived lipopolysaccaride (LPS) (Difco, Detroit, MI) during the final 24 hours of culture unless otherwise noted.

To distinguish between the presence of soluble factors or membrane-bound factors produced by the BM cells, 6-well transwell plates (0.4 um filter; Costar) were used. Various concentrations of feeder cells (whole BM) were added to the upper chamber, and the lower chamber contained 6×10^5 BM cells at 2×10^5/ml (low cell density BM). The DC were harvested from the lower chamber after 9 days of culture. In some experiments, FL

was excluded, or replaced with 100 ng/ml stem cell factor (SCF [Immunex], also known as c-*kit* ligand).

Cultures for cytokine neutralization studies were established as above, and neutralizing antibodies were included at the initiation of culture. Rat anti-murine IL-6 monoclonal antibody (mAb) (MP5-20F3) (Pharmingen, San Diego, CA) and the rat IgG_1 isotype control (Immunex) were used at 1 mg/ml. Rabbit anti-murine GM-CSF polyclonal antiserum (Immunex) was used at a dilution of 1:400, which was sufficient to neutralize 10 ng/ml of recombinant murine GM-CSF (data not shown).

Splenic-derived DC. Adult BALB/c or C57/BL/6 female mice were administered 10 mg FL/daily for 10 days in endotoxin-free PBS (Gibco) via intra-peritoneal injections.[3] Twenty-four hours after the last injection, the spleens were harvested and the DC enriched as previously described.[17] Briefly, spleens were digested with 100 units/ml collagenase (Worthington Biochemical Corp., Freehold, NJ), in Hanks' buffered salt solution (HBSS, Gibco) with Ca^{++} and Mg^{++} and 1% FBS for 30 minutes at 37°C. The spleens were minced and large debris filtered out before washing the single cell suspension in HBSS without Ca^{++} and Mg^{++} and 10 mM EDTA. For functional assays (MLR and stimulation of OVA-specific T cells) DC were enriched by running over a Nycodenz discontinuous gradient. Cells within the interface were collected and washed twice before using in antigen presenting cells (APC) function assays. There were less than 1% contaminating T cells or B cells from the DC-enriched interface.

Cytologic assays. Harvested cells were centrifuged at room temperature onto slides at 30-40,000 cells/slide. Slides were air dried and stained with LeukoStat (Fisher Diagnostics, Pittsburgh, PA) for morphological analysis. Phase-contrast observations of cultures were made using an inverted microscope (Nikon) at 400x magnification.

Flow cytometric analysis. DC from harvested cultures were centrifuged once and resuspended in cell staining medium (SM) consisting of PBS supplemented with 2% heat-inactivated calf serum (Gibco), 2% heat-inactivated mouse serum (Biocell, Rancho Dominguez, CA), 10 mg/ml 2.4G2 anti-Fc receptor mAb (ATCC, Rockville, MD), and 0.02% sodium azide (Sigma, St. Louis, MO). Cells were blocked with SM at 4°C for 20 minutes before incubation with mAbs. Cells were incubated with directly conjugated mAbs for 25 minutes at 4°C at $3\text{-}5\text{x}10^5$ cells per sample in a 50 ml volume. All mAbs were purchased from Pharmingen, except where noted. The following mAb (clone name) were used: CD1d (1B1), CD8a (53-6.7), CD11b (M1/70), CD11c (HL3), CD14 (rmC5-3), CD25 (7D4), CD40 (HM40-3), CD80 (16-10A1), CD86 (GL-1), IA^b (AF6-120.1), Gr-1 (RB6-8C5), Ly6C (AL-21), Ly6A/E (also known as Sca-1) (D7), and c-*kit* (ACK45). Phycoerytherin-conjugated F4/80 (CI:A3-1) mAb was purchased from Caltag Laboratories (Burlingame, CA). Biotinylated 33D1 (ATCC) and DEC205 (NLDC145; Accurate Chemical and Scientific Corp., Westbury, NY) binding were detected with streptavidin-Phycoerytherin (Molecular Probes, Eugene, OR). Propidium iodide, (Boehringer Mannheim, Indianopolis, IN) at 2 mg/ml was added to exclude dead cells from analysis. Ten thousand events per sample were collected. Flow cytometric analysis

was performed on a FACSCalibur (Becton-Dickinson, Mountain View, CA) using CELLQuest software (Becton-Dickinson). Gates were determined using appropriate isotype controls. Results are given as percent positive minus background from appropriate isotype controls.

Generation of DC from sorted progenitor cells. BM cells from adult female C57BL/6 mice were processed as above, but resuspended in SM after lysis of RBCs. Depletion of lineage positive cells was accomplished by incubating cells with unconjugated mAb to CD11b, CD122 (TMb1), B220 (RA3-6B2), Gr-1, NK1.1 (PK136), TER-119, and F4/80, for 25 minutes at 4°C. All antibodies were from Pharmingen, except F4/80 which was purchased from Caltag Laboratories. Labeled BM cells were washed, then incubated with sheep-anti-rat IgG conjugated magnetic beads (Dynal Inc.) per instructions. Non-adherent cells were collected and washed, then stained with Phycoerythrin-labeled anti-flt3 (A2F10.1) mAb and pooled FITC-labeled mAb to lineage markers (Pharmingen): CD3e (500A2), CD11b, CD11c, B220, Gr-1, and Ly6c, for 25 minutes at 4°C. The $flt3^+$, lineage negative population was sorted on the FACsVantage (Bection-Dickinson). Collected cells were cultured in a 200 ml volume/well at high density (approximately $2.5x10^5$ cells/ml) in U-well, 96-well plates (Costar). Media consisted of CM supplemented with 200 ng/ml FL and 10% conditioned medium from cultured spleen cells. Spleen cell-conditioned media, a source of DC-promoting activity[18], was prepared by mincing spleens from adult C57BL/6 mice in PBS, pipetting vigorously to break up clumps, then washing and resuspending cells in CM. Spleen cells were then cultured at $3x10^6$/ml in T-75 flasks (Costar) for 10-14 days before collecting the conditioned media. After culturing sorted progenitor cells for 2 days, each well was transferred to another well in a 24-well plate (Costar) containing FL and 10% conditioned media from spleen cells as above. Cultures were stimulated with 1 mg/ml E. coli-derived LPS on day 8, and harvested for cell counts and flow-cytometric analysis on day 9.

Mixed lymphocyte reaction (MLR) assay. DC were generated as described above. Briefly, BM cells from C57BL/6 mice were cultured for 9 days in media containing FL alone, or in cultures containing FL for 9 days to which LPS or IFNa was added during the final 24 hours of culture, or in cultures containing GM-CSF plus IL-4 for 7 days. Additionally, DC were enriched from the spleens of mice treated with FL for 10 days. T cells were enriched from spleen and peripheral lymph nodes (LN) of DBA/2 mice by depletion of non-T cells with mAbs against B220 and CD11b, then removal of mAb-coated cells with sheep-anti-rat IgG conjugated magnetic beads (Dynal). DBA/2 T cells at $1x10^5$ cells/well were seeded into U-well 96-well plates (Costar) and cultured with varying numbers of allogeneic C57BL/6-derived DC in 200 ml/well CM. Cultures were maintained for 5 days in CM at 37°C in a humidified atmosphere containing 5% CO_2 in air. Alamar blue (Biosource, Camarillo, CA) at 20 ml per well was added during the final 24 hours of culture.[19] The optical density of the assay plates were read at a wavelength of 570nm-600nm on a Molecular Devices plate reader (Sunnyvale, CA) using SoftMax software (Molecular Devices).

Preparation and purification of antigen-specific CD4 T cells. $CD4^+$ T cells recognizing a

peptide of chicken ovalbumin (OVA) in the context of IA^d were isolated from the spleens and peripheral LN of D011.10 TCR transgenic BALB/c mice.[20] Single cell suspensions were incubated for 30 minutes at 4°C with anti-CD8a (53-6.7), MHC Class II (IA^d, AMS-32.1), and B220 (RA3-6B2) (Pharmingen). $CD4^+$ T cells were enriched by depleting mAb-coated cells with sheep-anti-rat IgG conjugated magnetic beads (Dynal).

Antigen presentation assay. OVA presentation assays were performed in 96-well U-well plates (Costar). Naive D011.11 OVA-specific $CD4^+$ T cells at $1x10^5$/well were incubated with either a constant number of DC ($2x10^4$ cells) per well and titrated OVA protein (Calbiochem, San Diego, CA), or in a constant amount of OVA protein (300 mg/ml) with varying numbers of DC per well. DC were generated as described above. Briefly, BM cells from female BALB/c mice were cultured for 9 days in media containing FL alone, or in cultures containing FL for 9 days to which LPS or IFNa was added during the final 8 hours of culture, or in cultures containing GM-CSF plus IL-4 for 7 days. Additionally, DC were enriched from the spleens of mice treated with FL for 10 days. Cells were cultured in 200 ml/well modified DMEM medium containing 10% FBS, 2-ME, and PSG, at 37°C in a humidified atmosphere containing 10% CO_2 in air. Cells were cultured for 5 days, then pulsed with 0.5 mCi ^{3}H-thymidine for 8 hours, and the cells were then harvested onto glass fiber sheets for counting in a gas-phase beta counter.

In vitro IL-12 production assay. Myeloid- and lymphoid-type DC from FL-supplemented BALB/c BM cultures were sorted and separated by their expression of CD11b and CD11c as shown in figure 7B using the EPICS ELITE (Beckman Coulter, Brea, CA). Sorted cells were cultured in CM supplemented with 20 ng/ml GM-CSF (Immunex), 20/ng/ml IFNg (PBL, New Brunswick, NJ), and 50 ug/ml Pansorbin (Calbiochem, San Diego, CA) at $1x10^6$ cells/ml for 40 hours. Culture supernatants were assayed for murine IL-12 p70 using an ELISA Quantikine kit (R and D systems, Minneapolis, MN). Limit of detection was 8 pg/ml.

Statistical Analysis Statistical analyses were performed using the unpaired two-tailed Student's *t*-test.

Results

Generation of CD11c$^+$ DC from FL-supplemented BM cultures. It has previously been reported that the administration of FL to mice generates large numbers of DC in vivo.[3] We were interested in determining whether culture conditions could be established in which FL, as a single factor, could induce DC development in vitro. Murine BM cells were cultured at high density (1x10^6/ml), in the presence of 100 ng/ml FL. After 9 days, cultures contained small lymphoid-sized cells that grew in clusters associated with adherent macrophage-like cells (Fig 1). Cells derived from the FL-supplemented cultures of BM cells from BALB/c or C57BL/6 mice expressed variable levels of CD11b, CD11c, 33D1, CD86, MHC Class II, and Gr-1 (Fig 2). However, less than 1% were positive for CD19 (B cells), CD3e (T cells), NK1.1 (NK cells) or TER-119 (erythroid cells) based upon flow cytometric analysis (data not shown). The absence of lineage-specific, cell-surface antigens for T, B, NK, and erythroid cells, and expression of CD11c and 33D1, as well as low levels of MHC Class II and CD86 were suggestive of immature DC-lineage committed cells. Two populations of DC defined by CD11b expression were detected. One population expressed high levels of CD11b (CD11b^{bright}), while the other population expressed little to no CD11b (CD11b^{dull}). Most of the cells from FL-supplemented BM cultures were positive for CD11c (76%), with approximately 50% expressing CD86 or MHC Class II. Expression of 33D1, a marker of marginal zone DC[21], was restricted to the CD11b^{bright} population. Gr-1, a marker of granulocytes, was expressed on half of the CD11b$^+$ FL-derived cells from BALB/c cultures, but on only a low percentage (7%) of cells from C57BL/6 cultures. In mice administered FL for 9 days at 10 mg/mouse/day, we found that splenic derived CD11c$^+$ DC from C57BL/6 and BALB/c mice were 14% and 25% positive for Gr-1, respectively (data not shown). Thus, cells generated from FL-supplemented BM cultures from both C57BL/6 and BALB/c mice generate populations of cells that are phenotypically similar to DC subsets previously identified in lymphoid tissues of normal and FL-treated mice.[3,15]

FL, but not SCF, supports in vitro DC development. Unfractionated BM cells generated DC in cultures only when the seeding density exceeded 5x10^5/ml (data not shown). Therefore we investigated the role of endogenously produced soluble factor(s) in the generation of DC by using a transwell system that allows for the selective diffusion of soluble factors. BM cells at low cell density (2x10^5 cells/ml in lower chamber) were cultured with increasing numbers of BM cells (upper chamber) in the presence or absence of FL or stem cell factor (SCF), the ligand for c-*kit* which shares activities and structural homology to FL.[22] Regardless of the BM feeder cell density in the upper chamber, cells cultured at low cell density in the lower chamber did not survive in the absence of exogenous cytokines (Fig 3). Inclusion of SCF in the cultures resulted in a 2-fold expansion of cells in the lower chamber, which was further increased when feeder cells were included in the upper chamber. However, fewer than 4% of the cells from the lower chamber, generated in the SCF-supplemented cultures, expressed CD11c or MHC Class II, whereas 87% of the cells expressed high levels of Gr-1 and exhibited the morphology of immature granulocytes (data not shown). BM cells cultured at low density in FL-containing medium generated few cells when the BM feeder cells were excluded (25% of

the input number). However, there was a linear dose response in the generation of cells in the lower chamber when increasing numbers of BM feeder cells were included in the top chamber, demonstrating the importance of cell density for the production of soluble factor(s) (Fig 3). Cells harvested from the lower chambers under these conditions expressed CD11c (85%) and MHC Class II (70%). Thus, feeder cell-derived soluble factor(s) can support DC development in vitro when FL is the sole growth factor added exogenously.

Role of endogenous IL-6 in DC development from FL-supplemented BM cultures. We investigated the role of endogenous soluble factors in DC development in FL-supplemented cultures using neutralizing mAbs. Neutralizing mAbs against IL-2, IL-3, IL-4, IL-7, IL-11, IL-15, G-CSF, CSF-1 and TGF-b_{1-3} failed to inhibit DC development in vitro from FL-supplemented BM cultures (data not shown). Neutralizing mAbs against murine IL-6 inhibited approximately 70-80% of the DC development during 9 days of culture (Fig 4A). A similar effect was seen with neutralizing mAbs against the murine IL-6 receptor (IL-6R) and gp130, both components of the IL-6 receptor complex (data not shown). In addition, FL-supplemented cultures of BM cells from IL-6 gene KO mice produced fewer cells, compared with normal BM, and as expected were not affected by the inclusion of the neutralizing anti-IL-6 mAb (Fig 4B). Residual cells harvested from FL-supplemented cultures of BM from IL-6 gene KO mice, and control cultures where anti-IL-6 mAb was included still expressed CD11b, CD11c, CD86 and MHC Class II similar to control cultures (data not shown). BM cells cultured in recombinant murine IL-6 alone, did not generate DC (data not shown). Lastly, polyclonal antibody against murine GM-CSF did not affect DC development in this culture system (Fig 4A). Thus, IL-6, but not GM-CSF, is an important growth factor for DC development from FL-supplemented BM cultures.

Kinetics of DC development in FL-supplemented BM cultures. Cells from FL-supplemented BM cultures were harvested periodically over an 11-day period, counted and analyzed for cell-surface phenotype. Although the total cellularity was fairly constant over time (1-3x10^6 cells/well), there was a considerable change in the relative proportion of monocytic-myeloid and B-lymphoid cells, as assessed by flow cytometry (Fig 5). The number of cells expressing CD19 (B cells) and Gr-1 (primarily granulocytes) declined over time, while there was a concomitant increase in cells expressing CD11c and MHC Class II. After 9 days of culture there were no detectable erythroid cells (TER-119$^+$), T cells (CD3e$^+$) or NK cells (NK1.1$^+$) present (data not shown). Significant numbers of CD11c$^+$ cells were not present until 5-7 days of culture, and CD11c expression closely correlated with development of MHC Class II expression.

Activation of FL-derived DC. Since the DC generated from FL-supplemented cultures expressed low levels of DC-associated molecules (Fig 2), we concluded that DC with an immature phenotype were being generated. We investigated whether these cells could be activated in vitro with cytokines or LPS. DC generated from FL-supplemented BM cultures were treated with 1 mg/ml E. coli-derived LPS, 1000 units/ml IFNa, or 10 ng/ml GM-CSF to assess morphological and phenotypic changes associated with activation.

LPS, IFNa, or GM-CSF were added to FL-supplemented DC cultures during the final 24 hours of culture (day 8). There was no statistically significant change in cellularity in the cultures after 24 hours of stimulation with LPS, IFNa, or GM-CSF (data not shown). Cultures supplemented with FL alone contained small, lymphoid-sized cells, growing as single cell suspensions, although some cells grew in small clusters (<20 cells/cluster). Some of the cells also possessed short dendrites (Fig 6A). Cells from LPS-treated cultures were larger, formed clusters (>50 cells/cluster) that adhered to the plastic culture dishes, and when separated by gentle pipetting, exhibited long dendrites (Fig 6B). Cells from IFNa-treated cultures also increased in size and had extended dendrites, but did not form large adherent clusters (Fig 6C). FL-derived DC treated with GM-CSF for 24 hours formed large clusters and increased in size, but few cells possessed long dendrites (Fig 6D). In addition, for comparative purposes, we generated DC in cultures supplemented with GM-CSF and IL-4 as described.[16] BM cells cultured for 7 days in medium supplemented with 20 ng/ml GM-CSF and 20 ng/ml IL-4 consisted primarily of adherent giant-multinucleated cells and macrophage-like cells, and non-adherent cells consisting on monocytes and cells with dendriform bodies. Only those cells in the non-adherent fraction were used to compare with FL-derived DC (Fig 6E).

The cell-surface phenotype of activated DC from FL-supplemented cultures was assessed by flow cytometry. There was little change in expression of CD11b or CD11c after 24-hour treatment of cultures with LPS, IFNa or GM-CSF; however, there were notable differences in the expression of other cell-surface antigens. DC generated in FL alone did not express appreciable levels of CD25 (IL-2Ra), DEC205, CD1d, or CD8a, and relatively low levels of CD40, MHC Class II, CD86, and CD80 (Table 1, Fig 7A and 7B). LPS treatment resulted in the up-regulation of CD25, CD40, DEC205, MHC Class II, CD86, CD80, CD1d, and CD8a (Table 1 Fig 7A and 7B). CD8a and DEC205 were expressed primarily by the $CD11b^{dull}$ population (Fig 7B). In contrast, expression of c-*fms* (CD115), the receptor for CSF-1, decreased after LPS treatment (Table 1). Similar to treatment with LPS, IFNa increased the expression of CD25, CD40, DEC205, MHC Class II, CD86, CD80, CD1d, and CD8a, and decreased expression of c-*fms*. Surprisingly, although overnight treatment with GM-CSF resulted in up-regulation of CD40 and CD80, there was only a moderate increase in DEC205, MHC Class II, CD86, and CD1d (Table 1 and Fig 7A). In addition, unlike activation with LPS or IFNa, GM-CSF-induced no increase of CD8a cell surface expression, yet c-*fms* expression increased Table 1). 33D1 expression was restricted to the myeloid DC subset and there was little change in expression after stimulation with LPS, IFNa, and GM-CSF (Fig 7B and data not shown). Interestingly, many DC from unactivated cultures (FL alone) constitutively co-expressed c-*kit*, as well as Sca-1 (Stem cell antigen/Ly6A/E), and the expression of both cell surface antigens increased upon activation with LPS or IFNa (Table 1). Both c-*kit* and Sca-1 are cell-surface antigens typically used in murine stem-cell purification protocols.[23-25]

For comparative purposes we generated DC using BM cultures supplemented with GM-CSF plus IL-4 as previously described.[16] Flow cytometric analysis showed that most of the cells constitutively expressed high levels CD11b, CD11c, CD80, CD86, and MHC

Class II (Fig 7C). Some of the cells from these cultures also expressed CD1d, 33D1, CD25, CD40, DEC205, and Sca-1, but not c-*kit* nor CD8a (Fig 7C and Table 1). The phenotype of GM-CSF plus IL-4-derived DC did not change significantly after a 24 hour stimulation with LPS, IFNa or TNFa as previously reported (13 and data not shown). Thus, unlike DC generated in GM-CSF plus IL-4, cells generated in FL-supplemented BM cultures, when activated with LPS or IFNa overnight, express a phenotype similar to both lymphoid ($CD11b^{dull}/CD8a^{+}$)- and myeloid ($CD11b^{bright}/CD8a^{-}$)-type DC found in lymphoid tissues.

DC can be generated from flt3^{+}, lineage-depleted BM cell progenitors. To address the question of whether DC generated from FL-supplemented BM cultures arise from preexisting DC or progenitor cells, the following experiment was performed. Cells from the BM expressing flt3, a receptor found on lympho-hematopoietic progenitor cells[22], and lacking a number of lineage-specific markers (CD3e, CD11b, CD11c, CD122, B220, Gr-1, NK1.1, TER119, F4-80, and Ly6c) were purified. This $flt3^{+}$, lineage-depleted population represented less than 0.3% of whole BM. Sorted cells were cultured in FL plus conditioned medium from cultured-spleen cells which has previously been described to contain an IL-6-like activity and could promote murine DC development in vitro.[18] After 8 days of culture, the cells were stimulated with LPS and harvested 24 hours later. Addition of LPS was used to upregulate DC-associated markers as shown in figures 7A and 7B. Flow-cytometric analysis demonstrated two discrete populations as determined by levels of CD11b and CD11c (Fig 8). Similar to DC generated from whole BM cultured in FL and stimulated with LPS, most cells expressed high levels of MHC Class II, CD80, CD86, and CD40 (Fig 7A and 8). CD8a and 33D1 were expressed primarily by the $CD11b^{dull}$ and $CD11b^{bright}$ populations, respectively (data not shown). Thus, both myeloid-and lymphoid-type DC, as assessed by phenotype, could be generated from hematopoietic progenitors using FL and the conditioned medium from spleen cells.

FL-derived DC stimulate allogeneic T cell proliferation. DC from FL- or GM-CSF plus IL-4-supplemented BM cultures, as well as derived from the spleens of mice treated with FL for 10 days, were assayed for allo-stimulatory activity in an MLR assay. Each population of DC were approximately 75-80% positive for CD11c, and contained less than 1% contaminating T cells (data not shown). All populations of DC had potent allo-stimulatory activity, although those generated in vitro with FL alone had the least (Fig 9). BM cells cultured in FL, and stimulated for 24 hours with IFNa or LPS, had enhanced allo-stimulatory capacity over those in FL alone (approximately 10-fold, at 1000 DC/well). DC generated in BM cultures supplemented with GM-CSF plus IL-4 were comparable in activity to DC derived from cultures supplemented with FL and activated with LPS. Splenic-derived DC consistently exhibited less activity than in vitro-derived DC. Thus, FL-derived DC activated with IFNa or LPS, which induced upregulation of MHC Class II and costimulatory molecules, resulted in potent allo-stimulatory activity to induce T cell proliferation in an MLR assay.

Processing and presentation of OVA protein to OVA-specific CD4 T cells. We tested the capacity of both FL- and GM-CSF plus IL-4-derived DC to process OVA protein, and

present it to OVA-specific $CD4^+$ T cells in vitro. T cells from D011.10 transgenic mice express a TCRab specific for the OVA peptide fragment 323-339 presented on $I\text{-}A^d$ MHC Class II molecules.[20] OVA-specific $CD4^+$ T cells were enriched from spleen and LN of D011.10 mice, and then cultured with either varying numbers of DC in a constant concentration of OVA protein, or with constant numbers of DC with titrated OVA protein, and proliferation was measured after 5 days. DC were derived from in vitro cultures using FL (+/-LPS) or GM-CSF plus IL-4, as well as enriched from the spleens of mice treated with FL for 10 days. All populations of DC were approximately 72-76% positive for CD11c and contained less than 1% contaminating T cells (data not shown). DC derived in vitro with FL, and stimulated with LPS during the final 8 hours of culture, demonstrated similar activity as those DC from cultures supplemented with FL alone at inducing T cell proliferation when either DC were titrated, and OVA protein remained constant (300 mg/ml), or when OVA protein was titrated and APC remained constant (Fig 10A and 10B). FL-derived DC stimulated with IFNa for 8 hours were also used and found to have similar activity to those stimulated with LPS (data not shown). When OVA protein was held constant, and APC were titrated, DC generated in vitro using GM-CSF plus IL-4 demonstrated similar activity as FL-derived DC. Additionally, those DC generated in GM-CSF plus IL-4 were less stimulatory to T cells than FL-derived DC when DC were held constant, and the OVA protein was titrated (Fig 10B). Splenic-derived DC enriched from the spleens of mice treated with FL for 10 days were also used for comparison, but found to be consistently less stimulatory to T cell proliferation when compared to the in vitro-derived DC. These data demonstrate that DC generated in vitro with FL or FL plus IFNa are competent at processing and presenting OVA protein to OVA peptide-specific $CD4^+$ T cells, and were comparable to those DC generated in cultures supplemented with GM-CSF and IL-4.

IL-12 production is restricted to the lymphoid-type DCs. Among the most profound functional differences between myeloid- and lymphoid-type DCs is IL-12 production, which is limited to the lymphoid subset.[26-28] We asked whether sorted DCs generated in vitro from FL-supplemented cultures could produce IL-12 p70, and if so, would it be restricted to the $CD11b^{dull}$ lymphoid DC subset. Sorted DC subsets using the gates as shown in figure 7B, were cultured for 40 hours in the presence of SAC (pansorbin), GM-CSF and IFNg. Lymphoid-type DC generated from FL-supplemented BM cultures made 10-fold more IL-12 p70 than their myeloid DC counterparts (Table 2). No detectable IL-12 p70 was produced from unstimulated DC (data not shown).

Discussion

Here we describe a novel culture system to generate murine DC with cell-surface antigen expression and functional phenotype similar to those of DC residing in lymphoid tissues. This culture system requires the addition of a single growth factor, FL, whereas a structurally and functionally related growth factor, SCF, promotes the generation of immature granulocytes (Fig 3 and data not shown). Maximal DC numbers are achieved after 9-10 days of culture (Fig 5), which is similar to the kinetics of FL-induced DC expansion in vivo.[3] The cell density dependence of this culture system suggests the involvement of endogenous factor(s) as well. Rasko et al[29] have demonstrated the cell density dependence of murine BM cells to form colonies in response to FL alone, which suggests the presence of endogenous factors. Addition of neutralizing mAb to IL-2, IL-3, IL-4, IL-7, IL-11, IL-15, CSF-1, G-CSF, GM-CSF, or TGF-b_{1-3} to the FL-supplemented BM cultures does not inhibit DC development, whereas neutralizing anti-IL-6 mAb significantly decreases DC yields, and BM cultures from IL-6 gene KO mice generate fewer DC than the wild-type cultures (Fig 4). Additionally, neutralizing mAb to IL-6R and gp130 are inhibitory (data not shown). Interestingly, residual cells harvested from cultures where neutralizing anti-IL-6 or IL-6R mAb were included had similar cell-surface marker expression for CD11b, CD11c, CD86, and MHC Class II (data not shown). This suggests that IL-6 acts as a DC-proliferative factor, and is not necessary for DC differentiation in this in vitro culture system. These findings are consistent with results from previous studies demonstrating a role for IL-6 in DC development. Human $CD34^+$ progenitors driven towards DC development with GM-CSF, SCF and TNFa produce IL-6 in vitro and require IL-6 for optimal DC expansion.[30] In addition, a recently described culture system for the generation of murine DC from cultured spleen cells also exhibited an IL-6-like activity.[18] More recent data from our lab suggest that the IL-6 activity comes from the lineage negative population in BM (data not shown). This would be consistent with a previous report demonstrating lineage negative progenitor cells from murine BM cells as a source of IL-6.[31] The mechanisms by which IL-6, and perhaps other gp130-stimulating growth factors like IL-11, LIF and Oncostatin-M, affect DC development in vivo remains to be determined.

GM-CSF has the capacity to induce both DC development from precursor cells, as well as activation of DC in vitro and in vivo.[5,6,11,32-43] In the present study, GM-CSF was not required for DC generation from mouse BM in vitro (Fig 4), which is similar to a previous report that anti-GM-CSF mAb does not inhibit DC generation from murine thymic precursors cultured in a cytokine cocktail (TNFa, IL-1b, IL-7, SCF and IL-3).[14] Lymphoid tissues from GM-CSF gene KO mice have normal DC numbers[8]; however, these mice exhibit a defect in antigen-specific T cell and B cell activation at the level of the APC, which could be rectified by an injection of exogenous GM-CSF with the immunogen.[9] Taken together, this suggests that the role of GM-CSF in acquired immunity may lie at the level of DC activation, survival, and/or migration to lymphoid tissues, rather than DC development.

We have demonstrated that as little as 24 hours exposure to GM-CSF caused these

immature DC to cluster, and upregulate expression of CD40 and CD80, but there was surprisingly little effect on MHC Class II, CD86, and other DC-associated antigens (7A and Table 1). Optimal stimulation of FL-derived DC with GM-CSF may require longer incubation periods or the addition of other pro-inflammatory cytokines. LPS and IFNa had more potent effects, including increased expression of cell surface antigens expressed on mature DC from lymphoid tissues and important for DC function (i.e. MHC Class II, CD40, CD80, CD86, DEC205, and CD1d) (Fig 7A, 7B and Table 1).[3,13,26,35,36] Similar effects can be elicited in mice within 6 hours of LPS injection, and this is accompanied by DC migration from the marginal zones to the T-cell areas of spleen.[37] IFNa has also been described as a potent DC activator.[38,39] IFNa, induced maturation of human DC generated in GM-CSF, TNFa, and IL-4 under serum free conditions.[39] Other cell-surface antigens, such as CD8a, CD25, Sca-1 and c-*kit*, whose function in DC biology is unclear, were also up-regulated by LPS and IFNa using the FL-based culture system (Fig 7A, 7B and Table 1).

$CD8a^+$ DC are found in murine thymus, spleen and peripheral LN.[15] We found that stimulation of immature DC with LPS or IFNa, but not GM-CSF, resulted in upregulation of CD8a, and this is the first report of in vitro-derived murine DC expressing this molecule (Fig 7B, and Table 1). A recent report has described CD8a upregulation on murine skin-derived Langerhan cells induced by culturing cells in GM-CSF plus CD40L.[40] Surprisingly though, these cells also expressed CD8b which we do not find on the BM-derived DC (data not shown). We also found that TNFa induced potent DC maturation by upregulating MHC Class II, CD80, and CD86, but not CD8a (data not shown). This may explain why investigators culturing murine DC from thymic precursors in the lymphoid cocktail, which contains TNFa, but not IFNa, found no CD8a expression.[14]

LPS and IFNa down regulated receptor c-*fms* expression (Table 1). The down regulation of this receptor for CSF-1 has been previously reported on human DC.[41,42] The results of Olweus et al suggest that $CD34^+/CD123^{bright}$ human DC progenitors are derived from the granulomonocytic pathway since this population initially expresses c-*fms*, and differentiates to $CD1a^+$/c-fms^- DC after culture for 5 days in IL-3 plus GM-CSF.[42]

The co-expression of both c-*kit* and Sca-1 following activation of DC is a novel observation (Table 1). Both of these cell-surface antigens have been previously described to be expressed on lymphoid tissue DC[15,32,43-47], but never together in the same report. Murine hematopoietic stem cells from C57BL/6 mice are described as lineage marker negative (such as B220, Gr-1, CD11b, CD3e, and NK1.1) and positive for Sca-1 and c-*kit*.[23-25] We found that after stimulation using LPS or IFNa, nearly all DC expressed high levels of both Sca-1 and c-*kit*, and this included the $CD11b^{dull}$ lymphoid-type DC subset. Therefore, some activated DC have the same phenotype as described for stem cells from C57BL/6 mice. As a result of this observation, we recommend that investigators purifying stem cells from C57BL/6 mice include DC-specific cell surface antigens (CD11c and MHC Class II) for complete lineage depletion.

We have also shown that, similar to FL-treated mice, both lymphoid- and myeloid-type DC, as defined by phenotype, are generated when BM is cultured in vitro with FL.[3,26] Myeloid-type DCs are characterized as CD11b^{bright}, CD11c^{+}, 33D1^{+} and CD8a^{-}, whereas lymphoid-type DC are CD11b^{dull}, CD11c^{+}, 33D1^{-}, and CD8a^{+}. The 33D1 antigen, which was originally described as a marker of cells within the marginal zones[21], was expressed exclusively by the CD11b^{bright} cells (Fig 2 and 7B). Pulendran et al previously demonstrated that 33D1 was restricted to the myeloid-type DC subset that were increased in the spleens of mice after administration of FL.[26] Conversely, we found that there was no expression of 33D1 on the CD11b^{dull} subset, but some of this population did express CD8a and DEC205 after activation with LPS and IFNa (Fig 7A, 7B, Table 1 and data not shown). In addition, both CD11b^{dull} and CD11b^{bright} subsets were generated from flt3^{+}, lineage-depleted BM cells cultured in FL plus conditioned medium from cultured spleen cells (Fig 8).

One of the pressing issues in murine DC development is whether myeloid- and lymphoid-type DC represent separate lineages. Numerous studies have addressed this issue by culturing progenitors in combinations of cytokines excluding GM-CSF, or by injecting defined progenitors directly into mice.[14,43,45] However, more recent reports have indicated that thymic- and splenic-derived lymphoid-type DC development is not associated with T cell development since Notch 1 KOs or mice deficient in both c-kit and IL-2Rg chain have thymic DCs, but no T cells, nor detectable T cell progenitors.[48,49] We have not yet been able to demonstrate that only one type of DC could be generated in the above culture using whole BM or highly purified progenitors, and in addition, we find both types are typically generated at ratio of 1:1 (Fig 8). This suggests that lymphoid- and myeloid-type DC progenitors are at an equal frequency, and require the same cytokines for development, or that development of a common progenitor into a lymphoid- or myeloid-type DC is a stochastic process.

DC generated in vitro from BM supplemented with FL and stimulated with LPS had similar allo-stimulatory activity in a MLR assay as those DC generated in GM-CSF plus IL-4 (Fig 9). In addition, those DC generated in FL plus IFNa or LPS had comparable activity to GM-CSF plus IL-4-derived DC to stimulate OVA-specific T cells to proliferate (Fig 10A and 10B, and data not shown). Surprisingly, DC generated in vitro with FL alone were just as active as those DC stimulated with IFNa. This may be a result of the DC becoming activated by the low levels of endotoxin that contaminate OVA protein (data not shown). We have found that as little as 50 pg/ml of E. coli-derived LPS would activate these DC to upregulate MHC Class II and other DC-associated markers (data not shown). Although we have found that FL and GM-CSF-derived DC demonstrate similar functional activity in the assays we employed, there were subtle phenotypic and morphological differences. Most notably, the DC generated in GM-CSF plus IL-4 were larger, have a higher level of autoflouresence, were constitutively activated (surface expression of MHC Class II, CD80, CD86, CD1d, and CD40), and lacked expression of c-*kit* and CD8a (Fig 7A, 7B, and Table 1).

Lastly, to address whether lymphoid-type DC generated from FL-supplemented BM

cultures represent those found in lymphoid tissues, we stimulated both DC subsets to produce IL-12 which has been described to be restricted to the lymphoid-type subset as defined by CD11b expression[26] or CD8a expression[27,28]. Administration of LPS or soluble *Toxoplasma gondii* tachyzoite extract to mice results in production of IL-12 p40 by DC found in T cell areas of the spleen, and these cells coexpress CD11c, CD8a and DEC205.[27] We found that lymphoid-type DC from BM cultures produced 10-fold more IL-12 p70 than their myeloid-type counterparts (Table 2). In addition, these data suggest that the differences in IL-12 production by DC is intrinsic to the subset, and not a result of the microenvironment from which the DC were derived. Surprisingly, the GM-CSF plus IL-4-derived DC, which are considered to represent myeloid-type DC, make IL-12 p70 (>1000 pg/ml) in response to LPS alone (data not shown and ref. 13). However, it has not been demonstrated that these in vitro-derived myeloid-type DCs represent the myeloid-DC subset found in lymphoid tissues in vivo.

The culture system described in this report will be useful for our understanding of DC development for their potential use as adjuvants for both infectious disease and in tumor biology.

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Table 1. Phenotype of FL- and GM-CSF plus IL-4-derived DC

Marker	No addition (FL alone)	FL + LPS	FL + IFNa	FL + GM-CSF	GM-CSF + IL-4
CD25	0¶	47	10	1	7
CD1d	4	54	13	11	21
CD40	18	92	80	45	34
DEC205	3	27	11	10	37
CD8a	7	45	20	4	0
F4/80	26	24	14	24	13
Ly6C	16	24	42	20	ND
Sca-1	61	92	95	69	17
c-*kit*	69	91	100	57	2
c-*fms*	17	4	6	35	ND

Cultures were established with BM cells cultured in FL for 9 days or GM-CSF plus IL-4 for 7 days from C57BL/6 mice as described in Materials and Methods. LPS, IFNa, or GM-CSF were added to FL-supplemented cultures on the eighth day of culture, and the cells were harvested 24 hours later for flow-cytometric analysis. Numbers are percent positive, after subtraction of background using the appropriate isotype controls as described in Materials and Methods. Detection of the above antigens was performed by flow cytometric analysis a minimum of 3 times, each time giving similar results.

¶, % positive.

ND, not determined.

Table 2. IL-12 p70 production by myeloid-and lymphoid-type DC

Population	IL-12 p70 (pg/ml)
myeloid-type DC	31±15
lymphoid-type DC	312±46

DC were generated from FL-supplemented BALB/c BM cultures as described in Materials and Methods. Cells were harvested after 9 days of culture in FL alone, then sorted for myeloid-type and lymphoid-type DCs using CD11b and CD11c to delineate these populations (same gates used in figure 7B). Sorted DC were then cultured in CM supplemented with GM-CSF, IFNg and SAC for 40 hours at a cell density of $1x10^6$/ml. Supernatants were assayed for murine IL-12 p70 using an ELISA kit. Results are the average ± S.D. of 3 separate experiments.

Figure Legends

Fig 1. Phase-contrast view of FL-supplemented C57BL/6-derived BM cultures after 9 days of culture. Note the refractile, non-adherent cells growing on the sparse, macrophage-like cell layer.

Fig 2. Flow-cytometric analysis of cells generated in FL-supplemented BM. BM cells from cultures from C57BL/6 or BALB/c mice were cultured at $1x10^6$ cells/ml in FL for 9 days (see Materials and Methods). Gates were determined using isotype controls. Analyses were performed a minimum of three times for each marker.

Fig 3. Cell output from FL versus SCF-supplemented low cell density BM cultures in the presence of titrated BM cells using transwell plates. BM cells at low density ($2x10^5$/ml) were cultured in the lower chambers of transwell plates, separated by a 0.4 mm filter from the titrated feeder cells in the upper chambers. Cells in the upper and lower chambers were cultured in either culture medium alone (n), 100 ng/ml of FL (s), or 100 ng/ml SCF (l) for 10 days, then harvested from the lower chamber, counted, and analyzed for expression of cell-surface antigens by flow cytometry.

Fig 4. DC development from BM cells cultured in FL requires endogenous IL-6, but not GM-CSF. (A) C57BL/6 BM cells were cultured in FL plus anti-mIL-6 mAb, anti-mGM-CSF mAb, or control antibodies for 9 days as described in Materials and Methods. Replicate wells (n=3) were harvested and scored for cellularity. (B) C57BL/6 or IL-6 gene KO BM cells were cultured in FL plus anti-mIL-6 or control mAb for 9 days as described in Materials and Methods. Replicate wells (n=3) were harvested and scored for cellularity and results presented as mean $\pm$ SD. Each experiment was performed a minimum of 3 times. *Significantly different from normal BM cells cultured in FL plus Rat mAb isotype control ($P<.05$).

Fig 5. Kinetics of DC development from FL-supplemented C57BL/6 BM cultures. BM cells were cultured in FL at $1x10^6$ cells/ml, and on days 3, 5, 7, 9 and 11 of culture, were harvested for enumeration of cells and analyzed for the expression of Gr-1 (s), CD19 (u), CD11c (n), and MHC Class II (l). Results are presented as the mean $\pm$ SD from three separate experiments. Day 0 data were generated from freshly isolated BM cells. Gates for flow-cytometric analysis were determined using isotype controls as described in Materials and Methods. Analysis was performed a minimum of 3 times for each cell surface marker.

Fig 6. Morphology of cells generated from FL-supplemented BM cultures. BM cells from C57BL/6 mice were cultured in (A) FL alone for 9 days, or stimulated for the final 24 hours of culture with the addition of (B) LPS, (C) IFNa, (D) or GM-CSF.

(E) "Myeloid" DC from BM cells cultured in GM-CSF and IL-4 for 7 days. Cells were harvested and cytospins prepared. Cytospun cells were photographed at 400x magnification.

Fig 7. Flow-cytometric analysis of activated BM-derived DC. (A) FL-supplemented BM cells from C57BL/6 mice were cultured for 9 days, and stimulated with either LPS, IFNa, or GM-CSF during the last 24 hours of culture. (B) 3-color analysis of FL-derived (+/- LPS) DC using CD11b and CD11c to define myeloid- and lymphoid-type DC. (C) DC generated in BM cultures supplemented with GM-CSF and IL-4 for 7 days. Gates were determined using isotype controls as described in Materials and Methods. Results shown are representative of >5 experiments.

Fig 8. Flow-cytometric analysis of $flt3^+$/$lineage^-$-derived DC. $Flt3^+$/$lineage^-$ cells were sorted from BM and cultured for 9 days in 200 ng/ml FL plus conditioned media from spleen cells. LPS at 1 mg/ml was added during the last 24 hours to induce maturation. Gates were determined using isotype controls as described in Materials and Methods. Results shown are representative of 5 experiments.

Fig 9. Comparison of allo-stimulatory activity of FL and GM-CSF plus IL-4-derived DC. DC were enriched from spleens of mice treated with FL, and generated from the BM of C57BL/6 mice cultured in GM-CSF plus IL-4 or FL, as described in Material and Methods. Splenic-derived DC from mice treated with FL for 10 days (□), BM-derived DC from cultures using GM-CSF and IL-4 (l), FL alone (s), FL plus IFNa (u, and dashes), or FL plus LPS (n) were cultured at various numbers in the presence of a constant number ($1x10^5$/well) of T cells from DBA/2 mice for 4 days. Alamar blue was added for another 24 hours before measuring optical density (O.D.). DBA/2 T cells alone gave a mean O.D. of -0.044 ± 0.031. Background O.D. from DC alone was subtracted from DC with T cells.

Fig 10. Comparison of antigen processing and presentation by DC generated from FL- or GM-CSF plus IL-4-supplemented BM cultures. (A) Splenic-derived DC from mice treated with FL for 10 days (n), BM-derived DC from cultures using GM-CSF and IL-4 (l), FL alone (s, and dashes), or FL plus LPS (u),were cultured with constant OVA protein (300 mg/ml) and $1x10^5$/well OVA-specific D011.10 T cells. (B) DC and T cells were cultured at constant concentrations ($2x10^4$ DC/well), and OVA protein was titrated. T-cell proliferation was measured on day 5 for both assays. Background counts from OVA-specific T cells cultured without DC in OVA protein (300 mg/ml) were <4000 cpm, and T cells without OVA protein and with DC ($2x10^4$ DC/well) were <1000 cpm. Background counts from DC alone were <1000 cpm. Data represented is the mean ± SD of triplicate wells, and was performed 5 times.

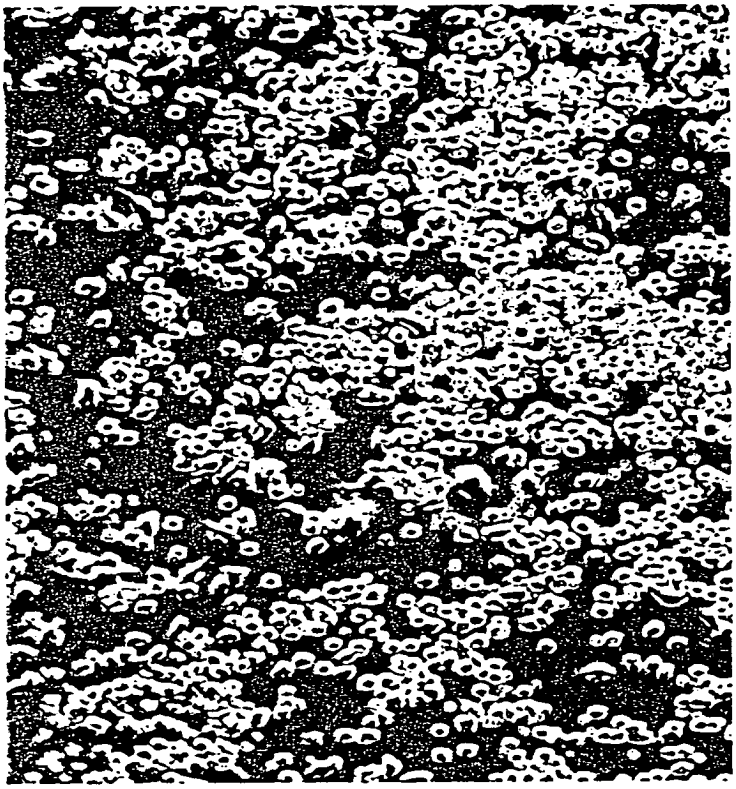


Fig 1

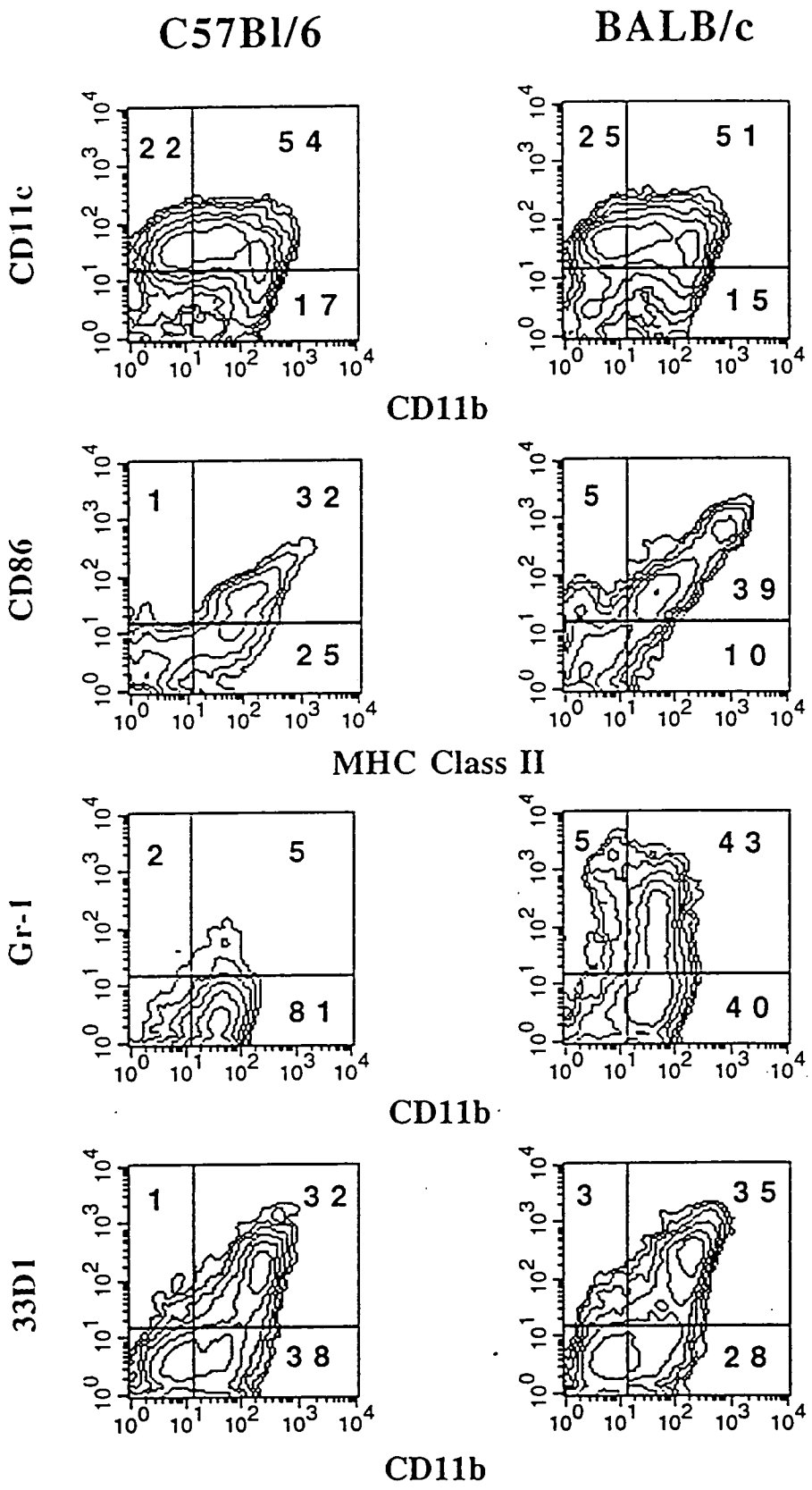

C57Bl/6
BALB/c
CD11c
CD11b
22
54
17
25
51
15
CD86
MHC Class II
1
32
25
5
39
10
Gr-1
CD11b
2
5
81
5
43
40
33D1
CD11b
1
32
38
3
35
28

Fig 2

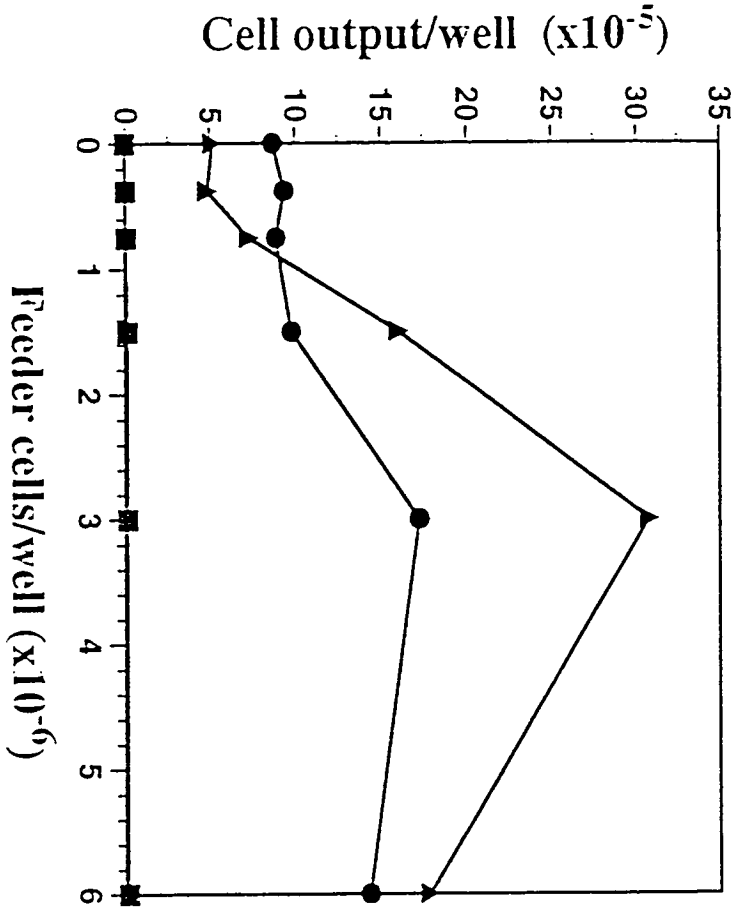

Cell output/well (x10⁻⁶)
0
5
10
15
20
25
30
35
Feeder cells/well (x10⁻⁶)
0
1
2
3
4
5
6

Fig 3

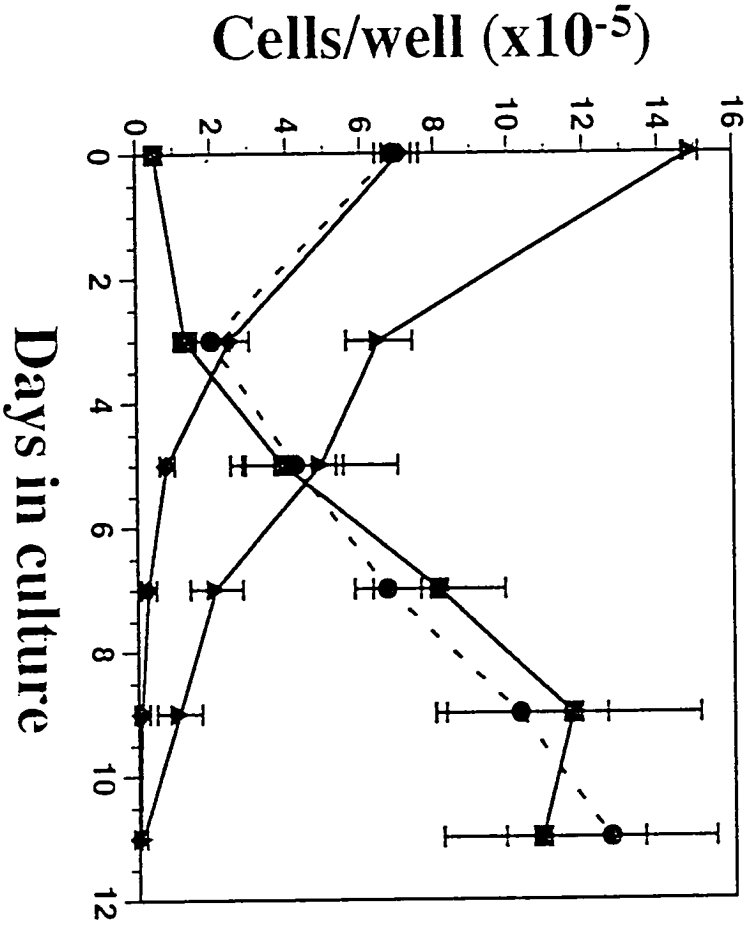
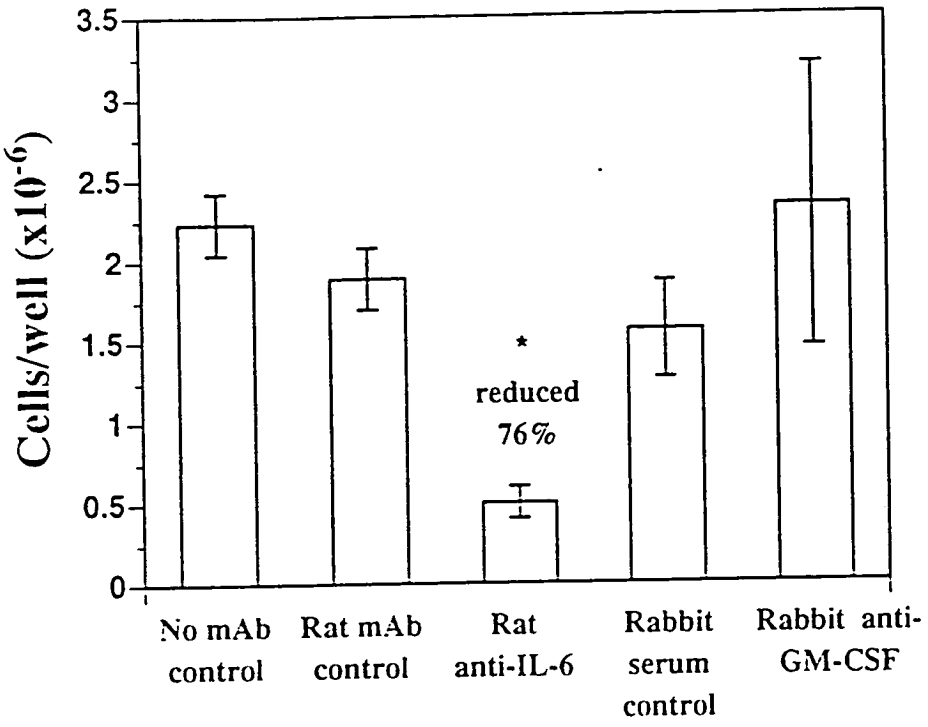


Fig 5

A.


Cells/well (x10-6)
3.5
3
2.5
2
1.5
1
0.5
0
*
reduced
76%
No mAb control
Rat mAb control
Rat anti-IL-6
Rabbit serum control
Rabbit anti-GM-CSF

B.

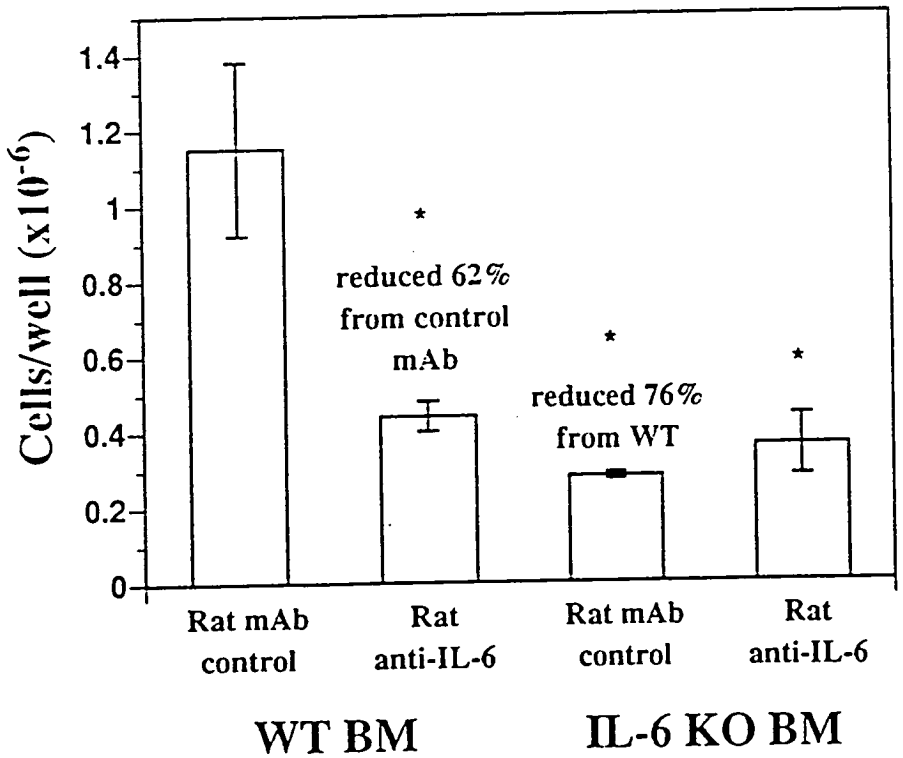

Cells/well (x10-6)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
reduced 62%
from control
mAb
*
reduced 76%
from WT
*
Rat mAb control
Rat anti-IL-6
Rat mAb control
Rat anti-IL-6
WT BM
IL-6 KO BM

Fig. 4

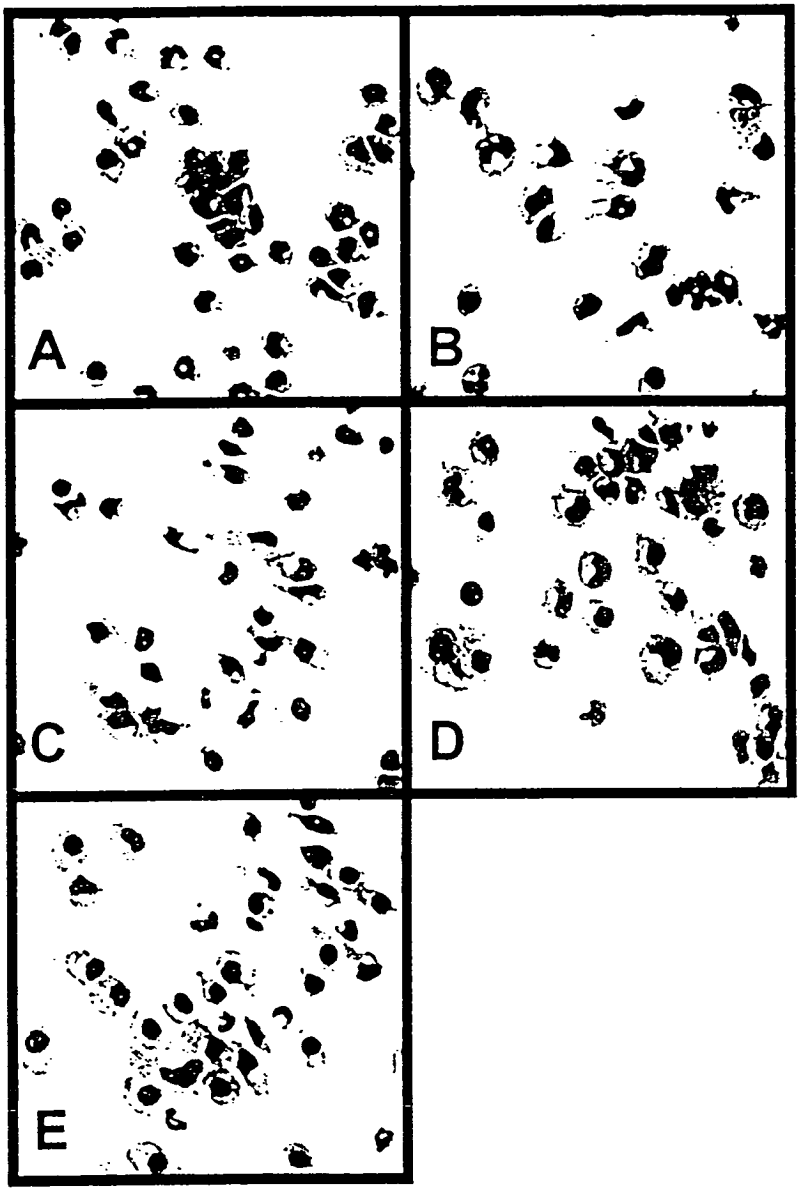


Fig 6

A.

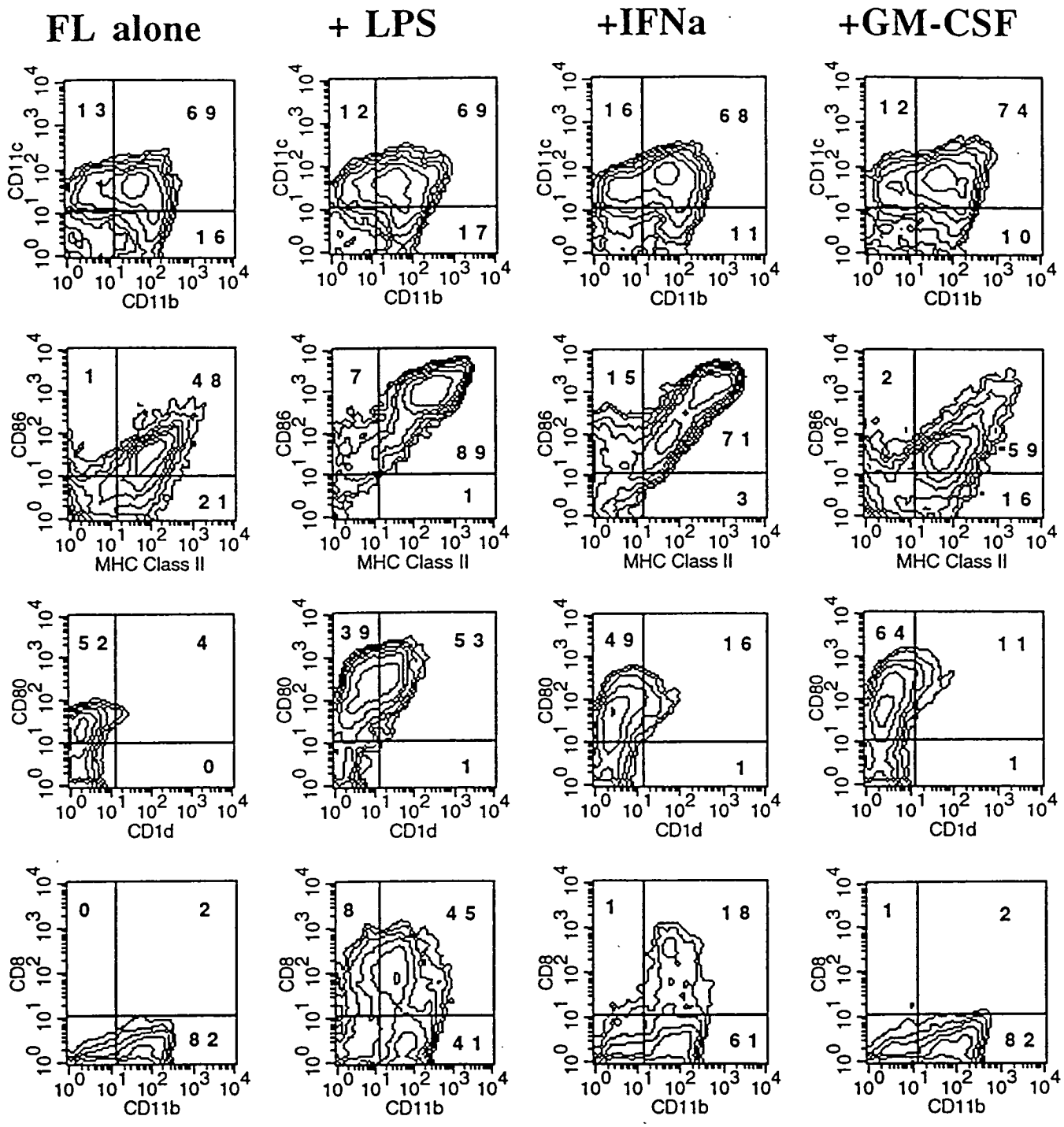
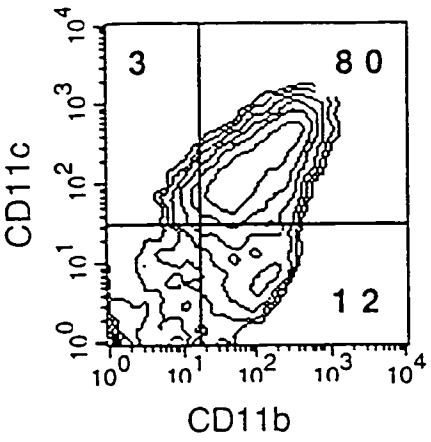
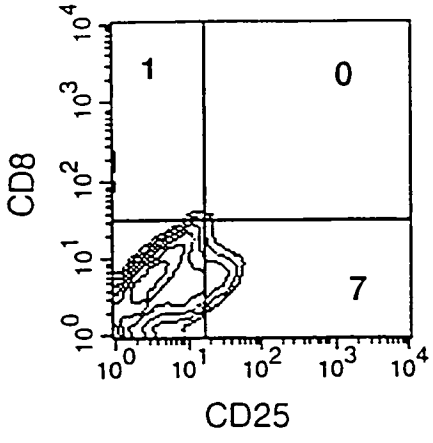

FL alone
+ LPS
+IFNa
+GM-CSF
CD11c
CD11b
CD86
MHC Class II
CD80
CD1d
CD8
13 69 16
12 69 17
16 68 11
12 74 10
1 48 21
7 89 1
15 71 3
2 59 16
52 4 0
39 53 1
49 16 1
64 11 1
0 2 82
8 45 41
1 18 61
1 2 82

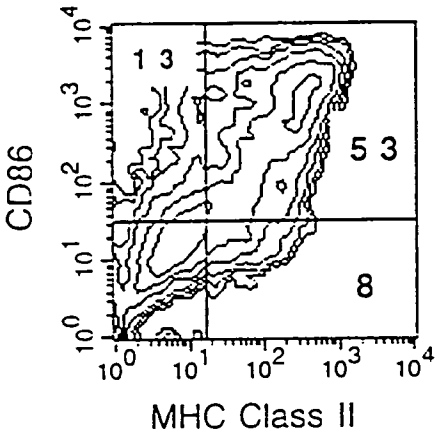
Fig 7A

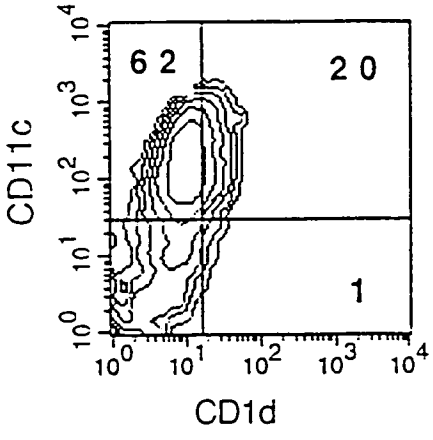
B.

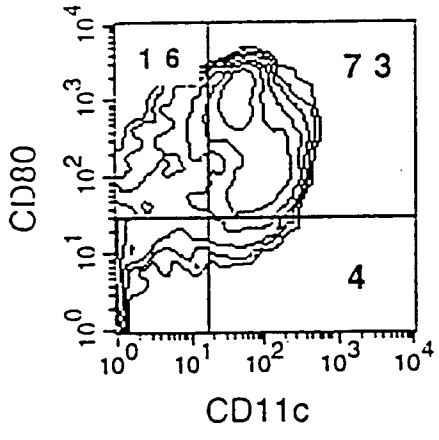
GM-CSF plus IL-4-derived DC











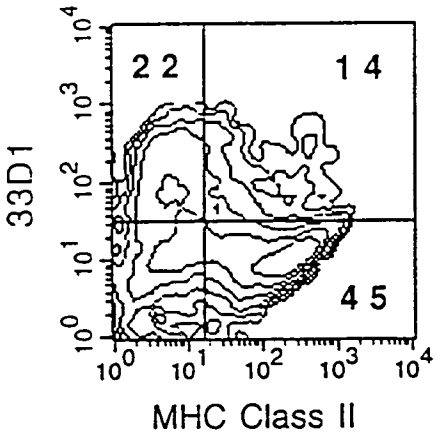


Fig. 7B

flt3 positive/lineage negative-derived DC

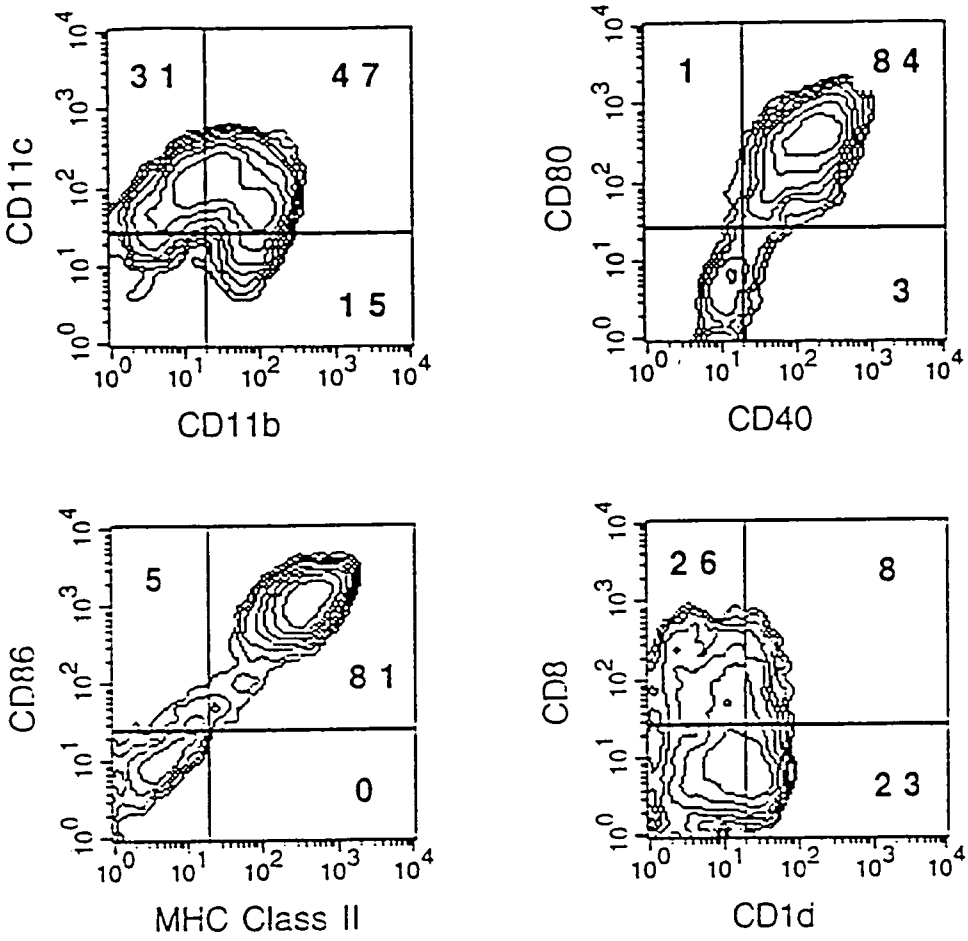


Fig 8

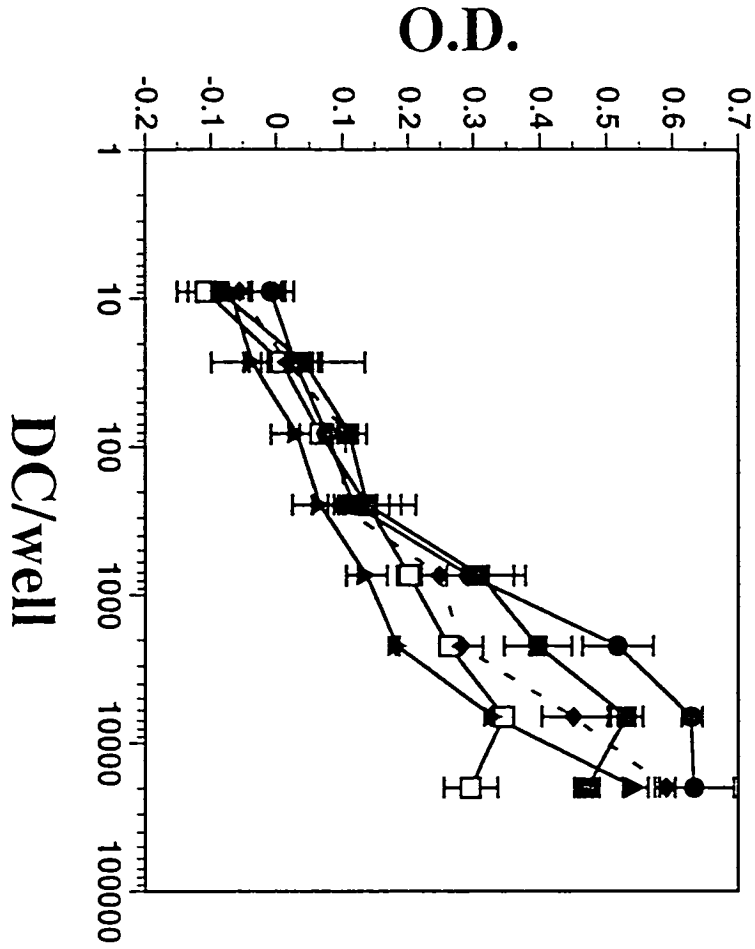
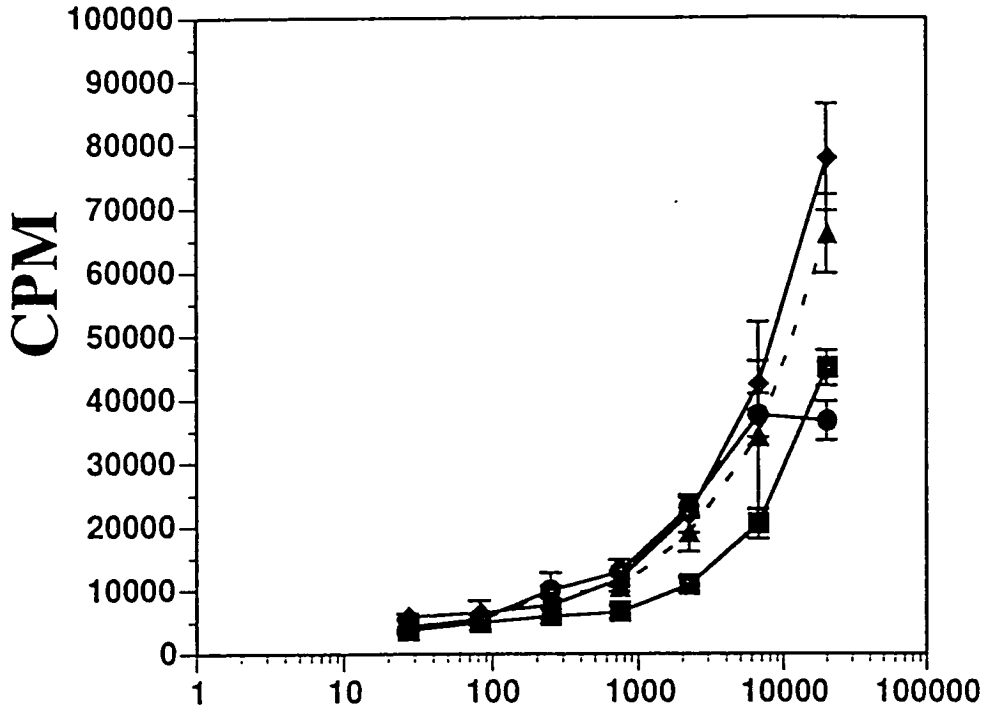


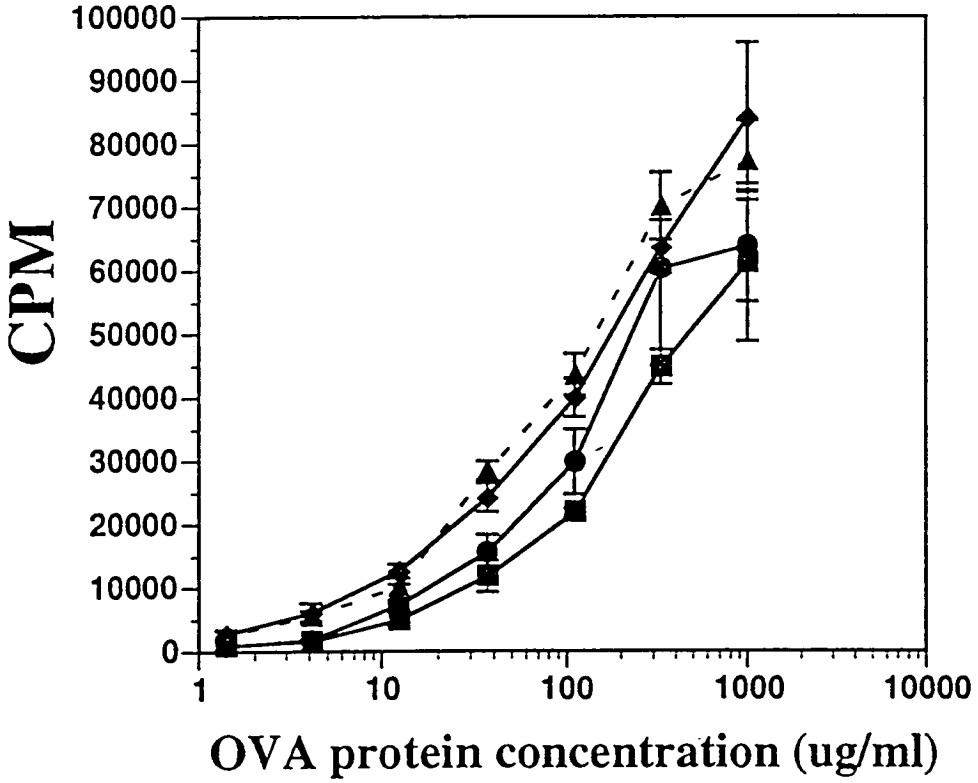
Fig 9

Fig 9

A.


CPM
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
1
10
100
1000
10000
100000
DC/well

B.


CPM
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
1
10
100
1000
10000
OVA protein concentration (ug/ml)